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Hypertension et régulation de l'expression moléculaire de l'angiotensinogène par la ribonucléoprotéine hétérogène nucléaire K

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Hypertension et régulation de l'expression moléculaire de l'angiotensinogène par la ribonucléoprotéine hétérogène nucléaire K

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Résumé

Le diabète est une maladie chronique dont la principale caractéristique est un niveau plasmatique élevé de glucose, qui est causé soit par un défaut dans la production d'insuline, l'action de l'insuline, ou les deux à la fois. Plusieurs études ont démontré que l'hyperglycémie chronique peut mener à la dysfonction et même la défaillance de plusieurs organes, dont le coeur, le système vasculaire, les yeux et les reins, se traduisant par des infarctus du myocarde, des accidents cérébro-vasculaires et des complications rétinales et rénales, respectivement. La néphropathie diabétique (DN) est la principale cause de déficience rénale et affecte près de 25-40% des patients diabétiques. La DN est invariablement associée à un risque élevé d'accident cérébro-vasculaire et de dysfonction cardivasculaire. L'angiotensinogène (Agt) est l'unique précurseur de tous les types d'angiotensines. En plus du système rénine-angiotensine (RAS) sytémique, le rein possède son propre système intrarénal et exprime tous les composants du RAS. L'Agt est fortement exprimé dans les cellules du tubule proximal rénal (RPTC) et y est converti en angiotensine II (AngII), le peptide biologiquement actif du RAS. Les patients diabétiques présentent de hauts niveaux d'AngII et une augmentation de l'expression des gènes du RAS, suggérant que l'activation du RAS intrarénal joue un rôle important dans la progression de la DN. Les mécanismes qui contrôlent la régulation du niveau rénal d'Agt par l'hyperglycémie et l'insuline demeurent mal compris.

Le but global de cette thèse est de mieux comprendre les mécanismes moléculaires qui contrôlent l'expression du gène *Agt* chez la souris Akita (un modèle murin de diabète de type 1). Dans cette optique, la première partie de la thèse se concentre sur deux facteurs de transcription de la famille des ribonucléoprotéines nucléaires hétérogènes (hnRNP). Chan et collaborateurs ont déjà identifié 2 protéines nucléaires hnRNP F et hnRNP K, de 48kD et 70kD respectivement. HnRNP F et hnRNP K forment un hétérodimère et se lient à l'élément de réponse à

l'insuline (IRE) présent dans le promoteur du gène *Agt* du rat et inhibent la transcription du gène *Agt* in vitro. Afin de déterminer si hnRNP F / K sont responsables de l'inhibition de l'expression rénale de *Agt* par l'insuline in vivo, nous avons étudié des souris Akita males traités ou non avec des implants d'insuline pour une période de 4 semaines. Des souris non-Akita males ont été employées comme contrôles. Les souris Akita développent de l'hypertension et de l'hypertrophie rénale. Le traitement à l'insuline rétablit les niveaux de glucose plasmatiques et la pression systolique (SBP), et atténue l'hypertrophie rénale, l'albuminurie (ratio albumine/créatinine urinaire, ACR) et les niveaux urinaires d'Agt et AngII chez les souris Akita. De plus, le traitement à l'insuline inhibe l'expression rénale du gène *Agt*, tout en augmentant l'expression des gènes *hnRNP F, hnRNP K* et *ACE2* (enzyme de conversion de l'angiotensine-2). Dans des RPTC in vitro, l'insuline inhibe Agt, mais stimule l'expression de hnRNP F et hnRNP K en présence de hautes concentrations de glucose, et ce via la voie de signalisation MAPK p44/42 (protéine kinase activée par un mitogène). La transfection avec des petits ARN interférents (siRNA) contre *hnRNP F* et *hnRNP K* prévient l'inhibition de l'expression d'*Agt* par l'insuline dans les RPTC. Cette étude démontre bien que l'insuline prévient l'hypertension et atténue les dommages rénaux observés chez les souris Akita diabétiques, en partie grâce à la suppression de la transcription rénale de *Agt,* via une augmentation de l'expression de *hnRNP F* et *hnRNP K*.

La seconde partie de cette thèse change de focus et se tourne vers le facteur Nrf2 (nuclear factor erythroid 2-related factor 2). Nrf2 est un facteur de transcription qui contrôle les gènes de la réponse antioxydante cellulaire en réponse au stress oxydant ou aux électrophiles. Le but de cette étude est d'examiner l'impact de la surexpression de la catalase (Cat) dans les RPTC sur l'expression du gène *Agt* via Nrf2 et sur le développement de l'hypertension et des dommages rénaux résultants chez les souris diabétiques Akita

transgéniques (Tg). Nos études ont démontré que la surexpression de Cat dans les souris Akita Cat-Tg normalise la SBP, atténue les dommages rénaux et inhibe l'expression des gènes *Nrf2* et *Agt* dans les RPTC. In vitro, le glucose élevé (HG) et l'oltipraz (un activateur de Nrf2) stimulent l'expression de *Nrf2* et *Agt*, et cet effet peut être bloqué par la trigonelline (inhibiteur de Nrf2), des siRNA contre *Nrf2*, des antioxydants ou des inhibiteurs pharmacologiques NF-κB et MAPK p38. La suppression de sites de réponse à Nrf2 présents dans le promoteur du gène *Agt* du rat abolit la stimulation par l'oltipraz. Finalement, des souris males adultes non-transgéniques traitées avec l'oltipraz montrent une augmentation de l'expression de *Nrf2* et *Agt* dans leurs RPTC et cette augmentation peut être normalisée par la trigonelline. Ces données permettent d'identifier un nouveau mécanisme d'action de Nrf2, par la stimulation du gène *Agt* intrarénal et l'activation du RAS, qui induisent l'hypertension et les dommages rénaux par le glucose élevé et les espèces réactives de l'oxygène chez les souris diabétiques. Nos conclusions permettent de démontrer que l'insuline induit l'expression de hnRNP F et hnRNP K, qui jouent ensuite un rôle protecteur en prévenant l'hypertension. La surexpression de la catalase dans les RPTC vient quant à elle atténuer l'activation de Nrf2 et ainsi réduit la SBP chez les souris Akita.

Mots-clés : rein, angiotensinogène, hnRNP F, hnRNP K, glucose, insuline, Nrf2, oltipraz, trigonelline, Akita, hypertension

Abstract

Diabetes mellitus is a chronic metabolic disorder characterized by high plasma glucose caused by an impairment of insulin production, insulin action or both. Accumulating evidence has shown that chronic hyperglycemia can lead to dysfunction and failure of multiple organs including the heart, vascular system, eyes, and kidneys resulting in myocardial infarction, stroke, and retinal and renal complications, respectively. Diabetic nephropathy (DN) is the leading cause of end-stage renal disease affecting approximately 25–40% of diabetic patients. DN is invariably associated with an increased risk of stroke and cardiovascular dysfunction. Angiotensinogen (Agt) is the sole precursor for all types of angiotensins. In addition to systemic renin-angiotensin system (RAS), all the components of the intrarenal RAS are expressed in the kidney. *Agt* is highly expressed in the renal proximal tubular cells (RPTCs) and converted into biologically active angiotensin II (Ang II). In Diabetics, intrarenal Ang II level and RAS gene expression are upregulated, suggesting that intrarenal RAS activation plays an important role in the progression of DN. The mechanism (s) underlying the regulation of renal Agt by hyperglycemia and insulin are not completely understood. The overall aim of this thesis is to understand the molecular mechanism(s) that regulate renal *Agt* gene expression in an Akita mouse (a mouse model of type 1 diabetes). For this purpose, the first part of this thesis focuses on two transcription factors from the heterogenous nuclear ribonucleoprotein (hnRNPs) family. Previously, Chan's group identified two nuclear proteins hnRNP F and hnRNP K of 48kD and 70kD, respectively. hnRNP F and hnRNP K form a heterodimer and bind to the insulin-responsive element (IRE) in the rat *Agt* gene promoter inhibiting *Agt* gene transcription in vitro. To determine whether hnRNP F / K mediate insulin inhibition of renal *Agt* expression in vivo, we used adult male Akita mice treated ± insulin implants for 4 weeks. Non-Akita mice served as controls. The Akita mice developed hypertension and exhibited

renal hypertrophy. Insulin treatment normalized plasma glucose levels and systolic blood pressure (SBP), attenuated renal hypertrophy, decreased urinary albumin/creatinine ratio (ACR) and urinary Agt and Ang II levels in Akita mice. Furthermore, insulin treatment inhibited renal *Agt* expression but enhanced *hnRNP F*, *hnRNP K* and angiotensin-converting enzyme-2 (*ACE2*) expression. In vitro, insulin inhibited Agt but stimulated hnRNP F and hnRNP K expression in high-glucose media via p44/42 mitogen-activated protein kinase signaling in RPTCs. Transfection with *hnRNP F* and *hnRNP K* small interfering RNAs (siRNA) prevented the insulin inhibition of *Agt* expression in RPTCs. This study demonstrates that insulin prevents hypertension and attenuates kidney injury, at least in part, through suppressing renal *Agt* transcription via upregulation of *hnRNP F* and *hnRNP K* expression in diabetic Akita mice.

In the second part of the thesis we focused on the nuclear factor erythroid 2-related factor 2 (Nrf2). Nrf2 is a transcription factor that regulates cellular antioxidant gene defense against oxidative stress or electrophiles. The purpose of this study is to investigate the impact of the overexpressing catalase (Cat) in RPTCs on Agt gene expression via Nrf2and the resulting effects on the development of hypertension and renal injury in diabetic Akita transgenic (Tg) mice. Our studies demonstrate that Cat overexpression normalizes SBP, attenuates renal injury, and inhibits RPTC *Nrf2* and *Agt* gene expression in the Akita Cat-Tg compared to Akita mice. In vitro, high glucose (HG) and Oltipraz stimulated Nrf2 and Agt gene expression; these changes were blocked by Trigonelline (an inhibitor of Nrf2), siRNA against Nrf2, antioxidants, or pharmacological inhibitors of NF-kB and p38 mitogen-activated protein kinase. Moreover, deletion of Nrf2-responsive elements in the rat Agt gene promoter abolishes the stimulatory effect of Oltipraz. Finally,non transgenic adult male mice treated with the Nrf2 activator Oltipraz, upregulated *Nrf2* and *Agt* expression in mouse RPTs, an effect that was

normalized by Trigonelline. These data identify a novel mechanism via which Nrf2 mediates the stimulation of intrarenal Agt gene expression and activates the RAS through whichHG/reactive oxygen species (ROS) induce hypertension and renal injury in diabetic mice. Our findings demonstrate that the insulin induced hnRNP F and hnRNP K gene expression play a protective role in the preventing hypertension. Catalase overexpression, in RPT's, attenuates Nrf2 activation and lowers the SBP in Akita mice.

Key words: Kidney, angiotensinogen, hnRNP F, hnRNP K, glucose, insulin, Nrf2, Oltiparz, trigonelline, Akita, hypertension.

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List of abbreviations

ACE	Angiotensin converting enzymes.
ACE2	Angiotensin-converting enzyme-2
ACEi	ACE inhibitors.
ACR	Albumin/creatinine ratio
AGEs	Advanced glycosylation end products.
Agt	Angiotensinogen
Ang I	Angiotensin I
Ang II	Angiotensin II
Ang IV	Angiotensin IV
Ang1–7	Angiotensin 1-7
APS	Pleckstrin homology.
AR	Aldose reductase.
ARBs	Angiotensin receptor blockers.
ARE	Antioxidant response element.
AT1R	Angiotensin II type 1 receptor.
AT2R	Angiotensin II type 2 receptor.
Cat	Catalase
CKD	Chronic kidney disease
CNC	Cap ‘n’ collar
CTGF	Connective tissue growth factor
Cul3	Cullin 3
DAG	Diacylglycerol.
DM	Diabetes mellitus.
DN	Diabetic nephropathy
ECM	Extracellular matrix.
eGFR	Estimated glomerular filtration rate
eIF4E	Eukaryotic translation initiation factor 4E
ER	Endoplasmic reticulum

ERK	Extracellular-signal-regulated kinases
ESRD	End stage of renal disease
GBM	Glomerular basement membrane
GDM	Gestational diabetes Mellitus
GFR	Glomerular filtration rate
Grb2	Growth factor receptor binder-2.
GSH	Glutathione
GSHPx	Glutathion Peroxidase.
gsk3	Glycogen synthase kinase-3.
GTFs	General transcription factors.
H&E	Hematoxylin and eosin
H2O2	Hydrogen peroxide.
HG	High glucose
hnRNP F	Heterogenous nuclear ribonucleoprotein F
hnRNPK	Heterogenous nuclear ribonucleoprotein K
hnRNPs	Heterogenous nuclear ribonucleoproteins
HO-1	Hemoxygenase-1
hrACE2	Human recombinant ACE2
IDF	Diabetes federation.
IGF-1	Insulin-like growth factor 1
IL-6	Interleukin-6
IR	Insulin receptor
IRE	Insulin-responsive element
IRR	Insulin receptor-related
IRSs	Insulin receptor substrates
JGA	Juxtaglomerular apparatus
KDOQI	Kidney disease outcomes quality initiative
Keap1	Kelch-like ECH-associated protein 1
MAPK	Mitogen-activated protein kinase.
Mapkk	Map kinase kinase.

Mapkkk	Map kinase kinase kinase.
MAS	Mas oncogene receptor
MAU	Microalbuminuria
NF-κB	Nuclear transcription factor κappa B.
NKF	National kidney foundation
NO	Nitric oxide.
NOD	The non-obese diabetic.
NOS	Nitric oxide synthase
NQO1	Quinone oxidoreductase 1.
Nrf1	Nuclear respiratory factor 1
Nrf2	Erythroid 2-related factor 2
PAS	Periodic acid schiff
PDK1	Phosphoinositide-dependant Kinase1.
PI3K	Phosphoatidylinositol 3-kinase.
PKC	Protein kinase C.
RAGE	Receptor of advanced glycosylation end products
RAS	Renin-Angiotensin System
RBPs	RNA-binding proteins
rMLC-2	Rat myosin light chain-2.
RNA Pol II	RNA polymerase II
ROS	Reactive oxygen species
RPTCs	Renal proximal tubular cells
SBP	Systolic blood pressure
SDH	Sorbitol dehydrogenase.
SH2	Src homology2.
siRNA	Small interfering RNA
SOD	Superoxide dismutases.
ssDNA	Single strand DNA
STZ	Streptozotocin
T1D	Type 1 diabetes mellitus

T2D	Type 2 diabetes mellitus.
TBM	Tubular basement membrane.
TBP	TATA-binding protein
TF	Transcription factors
TFBSs	Transcription factors binding sites
TGF-β	Transforming growth factor.
TNF-α	Tumor necrosis factor- α
TSS	Transcriptional start site.
UTR	Untranslated region.
Vitamin C	Ascorbic acid

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Chapter 1 : Introduction

1.1 Kidney physiology and histology

1.1.1 Renal physiology

The kidneys are paired retroperitoneal organs. In human adults, each kidney weighs about 150 g and is approximately 11-12 cm in length, 5.0-7.5 cm in width, and 2.5-3.0 cm in thickness [1]. The kidney's principal role is to regulate the composition and volume of the extracellular fluid by regulating water and electrolyte balance, osmotic and arterial blood pressure and also secreting the renin and angiotensin II hormones. The kidneys also regulate ion homeostasis in blood plasma maintaining the concentrations of sodium, chloride, potassium, and hydrogen independently. The kidneys excrete metabolic waste as by-products of metabolism, including urea, uric acid and creatinine. In addition, they produce specific hormones such as erythropoietin and the active form of vitamin D (1,25 -dihydroxy vitamin D3) while also playing an essential role in acid–base balance [2]. Table 1-1 shows the quantities of filtered and reabsorbed substances by the kidney in a 24 hr period.

Substance	Amount filtered (grams)	Amount reabsorbed (grams)	Amount in urine (grams)
Water	180 L	179 L	1 L
Proteins	10–20	10–20	0
Chlorine	630	625	5
Sodium	540	537	3
Bicarbonate	300	299.7	0.3
Glucose	180	180	0
Urea	53	28	25
Potassium	28	24	4
Uric acid	8.5	7.7	0.8
Creatinine	1.4	0	1.4

Table 1-1: Substances filtered and reabsorbed by the kidney per 24 hours [3].

1.1.2 Renal histology

The cross section of a kidney contains two regions, the cortex in the outer layer and the medulla in the inner layer. The cortex appears granular due to the spherical capillary tufts of the glomeruli. The glomeruli, convoluted tubules, and cortical collecting ducts are found in the cortex. The medulla has a striated appearance that results from the parallel arrangement of the loops of Henle, medullary collecting ducts, and blood vessels and can be divided into two regions: the outer and inner medulla [1, 2]. In a human kidney section, there are regional differences in the structure. The outer portion (cortex) contains all the glomeruli. The collecting ducts form a large portion of the inner kidney (medulla), giving it a striped, pyramid-like appearance, and these drains into the renal pelvis. The papilla is in the inner portion of the medulla [2]. See Figure 1-1.

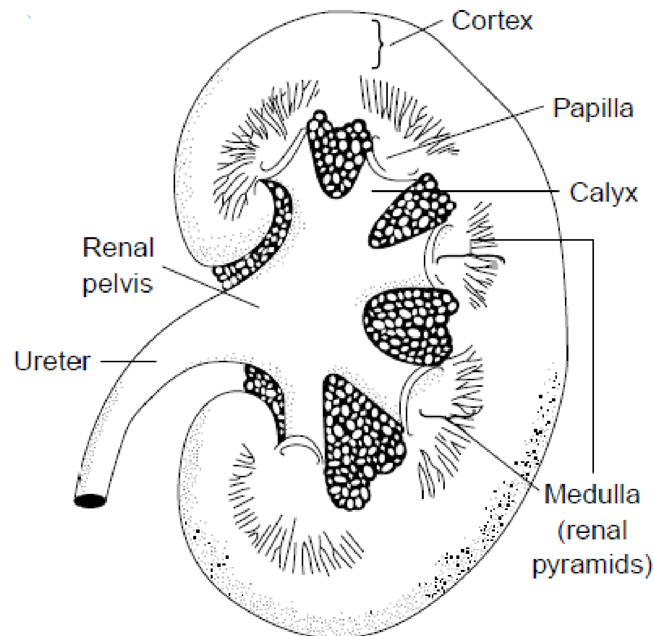


Figure 1-1: Schematic representation of a human kidney section shows regional differences. The outer portion (cortex) contains all the glomeruli. The collecting ducts form a large portion of the inner kidney (medulla), giving it a striped, pyramid-like appearance and these drain into the renal pelvis. The papilla is located in the inner portion of the medulla [2].

1.1.2.1 The nephron

The basic functional unit of the kidney is the nephron. Each nephron is an independent urine-forming unit. The number of nephrons per kidney is about one million in the human adult kidney and 30,000 in a rat kidney [1, 4]. Each nephron is capable of filtering blood, regulating the water volume and concentration of soluble substances, reabsorbing useful components, and excreting the rest. Three groups of nephrons are distinguishable, based on the location of their glomeruli in the cortex: the superficial, midcortical, and juxtamedullary nephrons. A nephron consists of two functionally different parts, the glomerulus and the tubules [2, 5].

1.1.2.1.1 Nephron components

A) The glomerulus

The renal corpuscle is the initial blood-filtering component of the nephron and is composed of a capillary network lined with a thin layer of endothelial cells (glomerulus). The glomerulus is a capillary network surrounded by the Bowman’s capsule. In the vertebrate kidney, the Bowman’s capsule is a double-walled, cup-shaped structure surrounding the glomerulus of each nephron [6] that serves as a filter for organic waste, excess inorganic salts, and water. The average diameter of the glomerulus is approximately 200 μm in the human kidney and 120 μm in the rat kidney [6]. The capillary structure of the glomerulus is permeable and increases the surface area for blood filtration [7].The mean area of filtration surface per glomerulus has been reported to be 0.203 mm^2 in the human kidney [8] and 0.184 mm^2 in the rat kidney [9]. The glomerular filtration barrier allows the filtration of small molecules but restricts the passage of macromolecules (e.g. plasma proteins). The glomerular filtration barrier consists of three layers: the endothelium or the lamina fenestra [3, 4], the basement membrane [5], and the podocytes with their slit diaphragms [6]. Podocytes are polarized, highly specialized and fully differentiated epithelial cells [5]. They line the urinary side of the

glomerular basement membrane (GBM), which functions as a fine filter permitting the permeability of molecules smaller than albumin. The glomerular ultrafiltrate passes into the urinary space of Bowman’s capsule. Fluid that comes out of the glomerulus is a plasma-like substance that flows into the renal tubule. The filtrate flows downstream the nephron through the tubule lumen, where tubular activity alters its composition and volume [6, 10].The appearance of protein in the urine indicates a compromised glomerular filtration barrier [6]. In diabetic nephropathy, proteinuria and microalbuminuria are used as clinical biomarkers [11]. Fig 1-2 shows renal corpuscle staining.

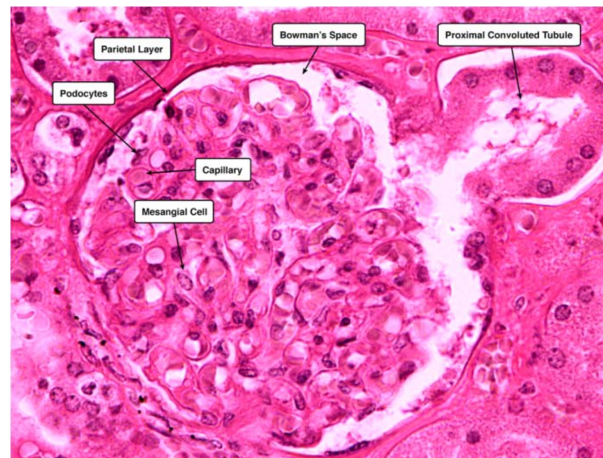


Figure 1-2: H&E-stained image of the glomerulus. Identified are the mesangial cells of the glomerulus and the podocytes of the visceral layer of Bowman’s capsule.http://medcell.med.yale.edu/histology/urinary_system_lab/renal_corpuscle.php.

B) Renal tubules

The renal tubule, which begins at and leads out of the Bowman’s capsule, is made up of a single layer of epithelial cells resting on a basement membrane. The renal tubules change the composition of filtrate by reabsorbing most of the useful organic nutrients and water that enter the tubular system. It also secretes additional waste products that do not enter with the ultrafiltrate. The tubule is divided into several components

including the proximal tubule, the descending and ascending loop of Henle, and the distal convoluted tubule and collecting duct. The cells in the tubular wall of each tubular region are structurally different and have distinct physiological properties [2]. A schematic representation of the nephron components is shown on Figure 1-3.

i) The proximal tubule

The proximal convoluted tubule comprises the first 60% of the proximal tubule [5]. It is about 10 mm in the rabbit [12], 8 mm in the rat, 4 to 5 mm in the mouse [13], and approximately 14 mm in the human. The most characteristic feature of the proximal tubules is the presence of an edge "brush" on the luminal surface of the tubules, which increases the surface area for reabsorption [14]. In the rat, three morphologically different segments have been identified [15]. The S1 segment is at the proximal end of the proximal tubule starting at the glomerulus and constitutes approximately two thirds of the pars convoluta. The S2 segment consists of the residual of the pars convoluta and the proximal component of the pars recta. The S3 segment contains the rest of the proximal tubule [6, 15]. The proximal tubule is responsible for reabsorbing all of the filtered glucose and amino acids and reabsorbing 70% of filtered solutes and water. It secretes various organic anions and cations [2]. Renal proximal tubular cells express all components of the RAS and overexpression of angiotensinogen increases tubular apoptosis in STZ-induced diabetes mice [16].

ii) The loop of Henle

The loop of Henle, connected to the proximal tubule is composed of different segments performing distinct functions: the descending limb, the thin and the thick ascending limb and the medullary and cortical thick ascending limb. The loop of Henle structure is responsible for the specific composition of aqueous ionic channels and urine concentration. As a

whole, it reabsorbs about 20% of the filtered sodium and chloride and 10% of the filtered water. The end of the loop of Henle contains cells of the macula densa, which monitor the sodium and chloride content of the lumen generating signals that influence renal function, specifically via the renin-angiotensin system [2].

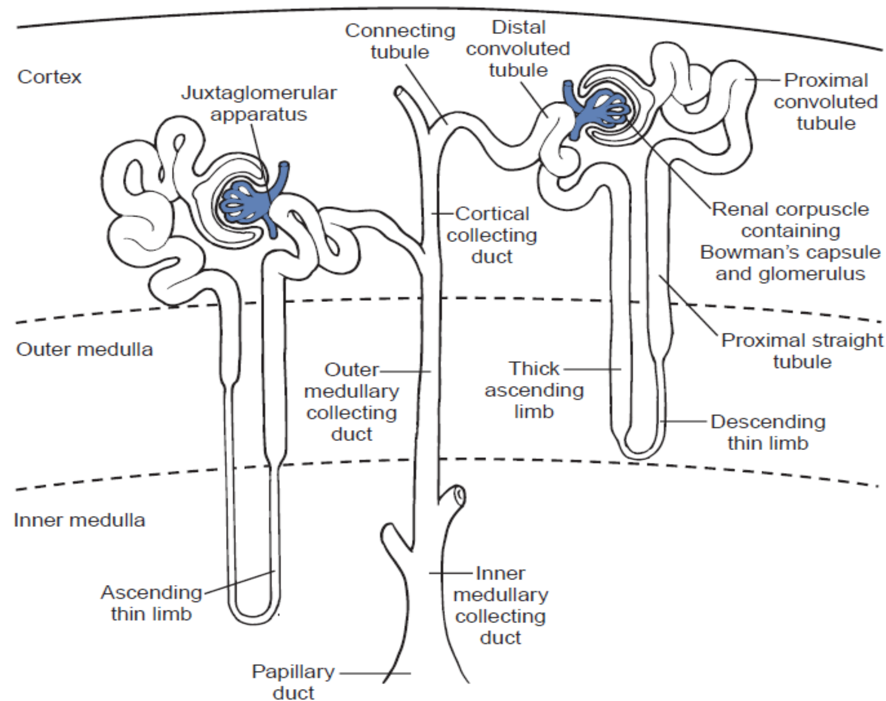


Figure 1-3: Components of the nephron and the collecting duct system. Onthe left is a long-looped juxtamedullary nephron;on the right is a superficial cortical nephron [5].

iii) Distal convoluted tubule

The distal nephron is considered to be the portion of the renal tubule beginning with the thick ascending limb of the loop of Henle and ending with the papillary-collecting duct. The distal convoluted tubuleis located immediately after the macula densa [17, 18]. The distal nephron is responsible for reabsorbing lower quantities of salt and water consisting of 9% of the filtered NaCl and 19% of the filtered water. Steep gradients for small ions and water can occur in the distal nephron. The distal

tubules are responsible for reabsorbing a certain amount of water and sodium ions. The aqueous channels in the apical and basal surfaces of these tubules, in turn, control the amount of reabsorbed substance [2].

1.2 Kidney disease

Kidney disease is associated with a significant reduction in both the length and quality of life [19]. Kidney dysfunction accelerates the onset and progression of cardiovascular disease, and eventually worsens its prognosis [20]. Kidney disease can arise from complications due to diabetes leading to diabetic nephropathy or from hypertension, widespread risk factors in the population. Other renal diseases include anatomic and metabolic kidney diseases and kidney damage due to certain nephrotoxic drugs. In chronic kidney disease (CKD) the damage in the kidneys decreases their ability to carry out their appropriate functions [21, 22]. The guidelines set forth by the Kidney Disease Outcomes Quality Initiative (KDOQI) define CKD as kidney damage occurring for 3 or more months caused by structural or functional abnormalities with or without a decreased Glomerular Filtration rate (GFR) [23, 24]. There are several factors that contribute to CDK but the most significant risk factors are diabetes and hypertension and are collectively responsible for up to 66% of the CKD cases. Hyperglycemia and high blood pressure cause damage to multiple organs including the kidney, heart, eye as well as the blood vessels. CKD eventually leads to End Stage Renal Disease (ESRD) characterized by kidney function of less than 10% of its full capacity [2]. CKD increases the rate of morbidity, mortality, and hospitalizations and at this stage patients require kidney transplants to survive.

1.2.1 Chronic Kidney Disease Prevalence

Patients with CKD are more likely to die of cardiovascular disease (CVD) and a minority progresses to ESRD [25, 26]. The prevalence of Stage 2-5 CKD has continued to increase since 1988 right along with increases in the prevalence of diabetes and hypertension, which are etiologic in approximately 40% and 25% of CKD cases aged more than 20 years old, respectively. Hypertension is triggered during stages 3–5 CKD while acid-base imbalance, dyslipidemia, and loss of glucose homeostasis occur later [27]. In Stage 4 CKD, death is a competing risk for progression to ESRD with about 17% of Stage 4 CKD progressing to Stage 5 [26]. CKD increases CVD morbidity and mortality risks in diabetics by 2- to 4-fold and in patients with hypertension and diabetes by 4- to 8-fold. Furthermore, CKD-attributable CVD risk increases several fold through stages 3–5 CKD. In 2010, the healthcare-associated costs for ESRD were $28 billion and expected to almost double to $54 billion by 2020 [27].

1.2.2 Laboratory Measurements for Kidney Disease

Kidney damage is defined by any one of the following laboratory tests: persistent proteinuria as well as estimated GFR (eGFR) less than 60ml/min/1.73 m^2 on two occasions separated by at least 3 months [28].

1.2.2.1 Assessment of Proteinuria

Assessment of albumin and/or protein excretion in the urine is a key step in the early detection and appropriate management of CKD. In the urine of healthy people, albumin is normally present in small quantities. The levels of albumin and other proteins rapidly rise when the kidneys are damaged, a phenomena referred to as proteinuria [29, 30]. Moreover, proteinuria tightly correlates with quantitative histological measures of interstitial fibrosis [31]. Microalbuminuria (MAU) refers to the increase in the concentration of urinary albumin that is indicative of either systemic or

renal malfunction. In CKD, the kidneys excrete more than 30 milligrams of albumin per gram of creatinine in their urine, regardless of the change in the eGFR [30]. In CKD, the albumin has to cross the glomerular filtration barrier under the effect of intraglomerular pressure increases, as well as the loss of the negatively-charged glycosaminoglycans in the cellular basement membrane, which lead to an enlargement in the pore size of the basement membrane and the leaking of albumin from the kidney to the urine [32]. Structural abnormalities that cause albuminuria are shown in Figure 1-4.

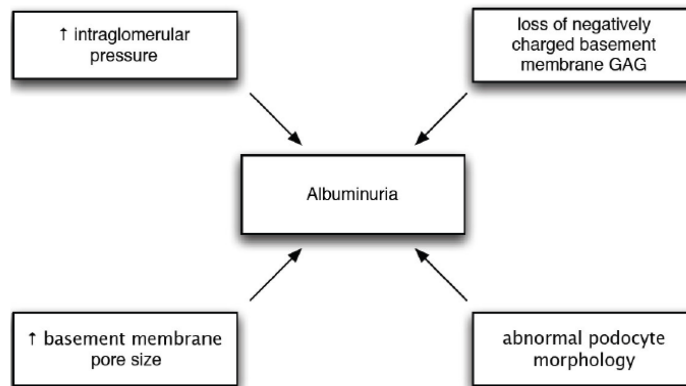


Figure 1-4: Structural abnormalities have an effect on albuminuria [32].

1.2.2.2 Estimation of Glomerular Filtration Rate (GFR)

The kidneys have a high blood flow. Glomerular filtration involves the ultrafiltration of plasma in the glomerulus [8]. The glomerular filtration barrier functions as a fine molecular sieve allowing small molecules to pass while restricting macromolecules such as plasma proteins. Kidney function can be tested by measuring the glomerular filtration rate (GFR) [33]. It is defined as the rate at which plasma is filtered by the kidney glomeruli [2]. Ideally, the GFR should be measured by inulin levels. Inulin has many advantages; it is filterable by the glomeruli, non-toxic, not reabsorbed or secreted by the kidney tubules, and it is detectable in both plasma and urine samples [34]. The National Kidney Foundation (NKF)

initiated the Kidney Disease Outcome Quality Initiative (KDOQI) to assist physicians in identifying, staging, and treating kidney disease [23]. The five stages are summarized in Table 1-2.

The five stages of chronic kidney disease

	STAGE 1	STAGE 2	STAGE 3	STAGE 4	STAGE 5
Amount of kidney function remaining at each stage	More than 90%	60 to 89%	30 to 59%	15 to 29%	Less than 15%
Description of each stage	Early kidney damage with normal or even increased function.	Worse kidney damage with reduced function.	Even worse kidney damage with less function.	Kidney damage is so severe with such poor function that the kidneys are barely able to keep the person alive.	End-stage Renal Disease: kidney function is severely impaired. The kidneys are not working well enough to keep the person alive.
Symptoms	No symptoms observed. Urea and creatinine levels are normal.	No symptoms observed. Urea and creatinine levels are normal, or mildly elevated.	Early symptoms occur and may include tiredness, poor appetite, and itching. Creatinine level rises, excess urea is present, and anemia may begin to occur.	Tiredness, poor appetite, and itching may get worse.	Symptoms may include poor sleeping at night, difficulty breathing, itchiness, and frequent vomiting. High levels of creatinine and urea are present.
eGFR (estimated Glomerular Filtration Rate)	90 ml/min or more	60 – 89 ml/min	30 – 59 ml/min	15 – 29 ml/min	15 ml/min or less
Treatment options*	Identify cause and try to reverse it.	Monitor creatinine level, blood pressure, and general health and well-being. Try to stop or slow the worsening kidney function.	Continue to try to stop or slow the worsening kidney function. Patient learns more about the disease and treatment options.	Plan and create access site for dialysis. Receive assessment for possible transplant.	Start renal replacement therapy: dialysis or transplantation.

Table 1-2: Stages of chronic kidney disease, as defined by KDOQI.

1.3 History of diabetes

Diabetes is a worldwide epidemic, and without effective prevention and management programs its rate will continue to globally increase. Diabetes was first mentioned by the ancient Egyptians. In 1874, the Egyptologist

Georg Ebers published one of the oldest medical documents written by an Egyptian, around 1530 BC, called "Ebers Papyrus" [35]. In it, there is mention of specific symptoms indicative of diabetes mellitus and a description of a condition of "too great emptying of the urine". For the treatment of this condition, ancient Egyptian physicians advocated the use of wheat grains, fruit, and sweet beer [36]. In 130–200 AD, the Greek physician Aretaeus noted a disease with symptoms of constant thirst, excessive urination and weight loss. He named the condition 'diabetes' meaning 'flowing through' [35]. The first clear reference to diabetes was made by an Arab physician, Avicenna (980–1037 AD), who described in detail the exact clinical features and complications of the disease and its progress [37]. Indian physicians developed the first clinical test for diabetes. They observed that the urine from people with diabetes attracted ants and flies. They named the condition "honey urine"[36]. In 1815, Eugene Chevreul proved that the sugar in urine of individuals with diabetes was glucose[36]. The diagnostic test developed by Von Fehling became an acceptable quantitative test for glucose in urine in 1848. As a result, in the nineteenth century, glucosuria became an accepted diagnostic criterion for diabetes. In the 20th century Bang, Folin, Lewis, Benedict, Shaffer and many others pioneered laboratory methods for quantitative blood sugar [35].

1.3.1 Prevalence of diabetes

Diabetes is on the rise worldwide and countries are struggling to keep pace. Over the past three decades, there has been an explosive increase in the prevalence of diabetes mellitus (DM). Diabetes mellitus is a complicated, chronic disorder characterized by either insufficient insulin production by the beta cells of the pancreas or by cellular resistance to insulin. Insulin insufficiency results in elevated blood glucose levels, or hyperglycemia [38]. According to the International Diabetes Federation (IDF), one in ten of the world's population will have diabetes by 2035.

Today, there are 382 million people living with diabetes. A further 316 million with impaired glucose tolerance are at high risk from the disease – an alarming number that is set to reach 471 million by 2035. IDF's most recent estimates show that people living with diabetes will surge from 382 million to 592 million people by 2035 [39]. As a result, individuals with diabetes are at greater risk for a number of disorders or complications, including myocardial infarction, cerebrovascular accident (stroke), blindness, kidney disease, and lower limb amputations. Diabetes complications are responsible for 5.1 million deaths and cost USD 548 billion in healthcare spending (www.idf.org/diabetesatlas).There is considerable data indicating that the chronic elevation of plasma glucose causes many of the major complications of diabetes, including nephropathy, retinopathy, neuropathy, and macro- and microvascular damage [40]. Insulin and oral antidiabetic drugs, along with diet and exercise, are the cornerstones of treatment for diabetes mellitus [41]. They are used to prevent episodes of hypoglycemia and to normalize carbohydrate metabolism. The new estimates show an increasing trend toward younger and younger people developingdiabetes, a trend that is troubling for future generations, Figure 1-5 (www.idf.org/diabetesatlas).

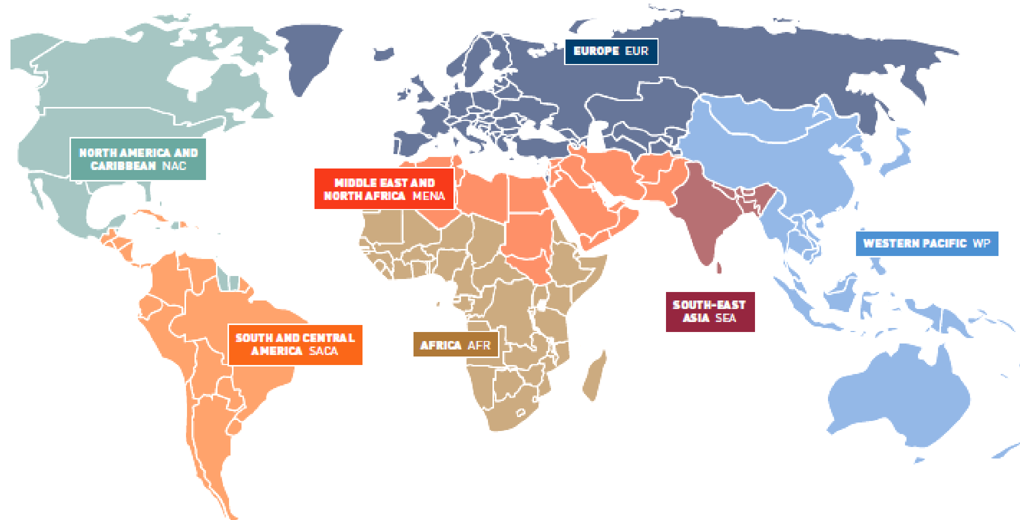
1.3.2 Types of diabetes mellitus

Diabetes encompasses a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action or both[38]. There are three major types of diabetes:

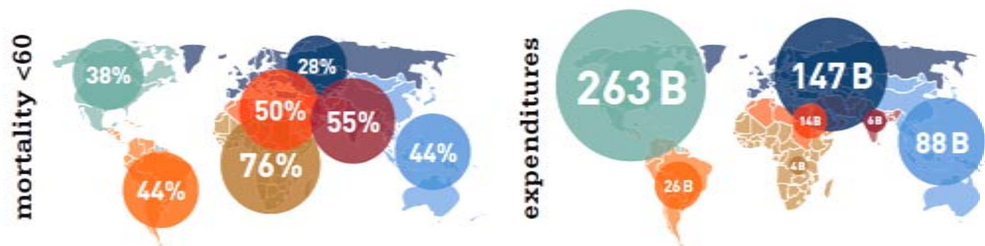
1.3.2.1Type 1 diabetes mellitus (T1D)

Previously, T1D was known as insulin-dependent diabetes. In T1D the pancreas does not produce adequate amounts of insulin to regulate glucose levels in the blood. Autoimmune destruction of the pancreatic beta cells causes the development of T1D. The immune system destroys the beta cells that produce insulin in the pancreas [42] and insulin therapy

is required for the treatment of this type of diabetes, the absence of which results in death.



IDF REGION	2013 MILLIONS	2035 MILLIONS	INCREASE %
Africa	19.8	41.4	**109%**
Middle East and North Africa	34.6	67.9	**96%**
South-East Asia	72.1	123	**71%**
South and Central America	24.1	38.5	**60%**
Western Pacific	138.2	201.8	**46%**
North America and Caribbean	36.7	50.4	**37%**
Europe	56.3	68.9	**22%**
World	**381.8**	**591.9**	**55%**



Proportion of deaths due to diabetes in people under 60 years of age, 2013

Health expenditure (USD) due to diabetes (20-79 years), 2013

Figure 1-5: IDF Regions and global projections of the number of people with diabetes (20-79 years), 2013 and 2035.

1.3.2.2 Type 2 diabetes mellitus (T2D)

T2D, formerlyknown as noninsulin-dependent diabetes is the most common type of diabetes, in which the pancreas does not produce enough insulin, or there is decreased sensitivity to insulin. It is usually seen in adults, but it is increasingly prevalent in children and adolescents [43]. The risk factors for developing diabetes are obesity, poor diet, physical inactivity, advancing age, ethnicity and high blood glucose during pregnancy,affecting the unborn child and genetics [43].

1.3.2.3 Gestational diabetes mellitus (GDM)

GDM occurs in a minority of pregnant women around the 24th week of pregnancy [44, 45] in whom a resistance to insulin and resulting high blood glucose occur. Gestational diabetes in mothers normally resolves itself after the birth of the child. However, women who have had gestational diabetes are at a higher risk of T2D later in life [46]. Gestational diabetes affects the babies by increasing their risk of developing T2D later in life [47].

1.3.3 Diabetic nephropathy (DN)

DN, a common diabetic complication, is a clinical syndrome characterized by progressive renal insufficiency in the setting of hyperglycemia, persistent albuminuria, hypertension, decreased GFR, and a highly-elevated risk of cardiovascular morbidity and mortality [48]. In most Western countries, diabetes has become the most common cause of kidney failure or end-stage renal disease. DN is a silent disease that usually manifests itself after 10 years' duration of T1D, but may be present at diagnosis of T2D [49]. Type 1 and type 2 diabetic subjects accounted for 44% of new cases of kidney dysfunction in 2008 [50]. Several factors along with diabetes contribute to inducing renal lesion damage, including increased systemic and intraglomerular pressure, as well as activation of the RAS and endothelin [51]. In DN, the most important structural

abnormality in the kidney glomerulus is thickening of the glomerular basement membrane (GBM) and mesangial expansion [52]. The GBM thickening change has been recognized as early as 1.5 to 2.5 years after the onset of type 1 diabetes [53], while the mesangial expansion can be detected 3.5 to 5.5 years later [54] . Thickening in the Bowman's capsule is also regularly present. The study done by Gambara et al. [53] shows that 33% of patients with proteinuria caused by type 2 diabetes had glomerulosclerosis. In addition to the abnormalities in the glomerulus, changes occurred in the tubules, which include thickening in the tubular basement membrane (TBM) and tubular atrophy [53].

Several mechanisms have been postulated for understanding the effect of hyperglycemia and tissue damage. These mechanisms are glucose-dependent pathways that include advanced glycosylation end products, increased polyol pathway flux, increased hexosamine pathway activity, oxidative stress and protein kinase C activation [55]. These lead to increased proteinuria, glomerulosclerosis and ultimately tubulointerstitial fibrosis. It has been shown that DN is one of the most significant long-term complications in terms of morbidity and mortality for individual patients with diabetes[55].

1.3.3.1 Role of podocytes in diabetic nephropathy

Podocyte injury has been demonstrated in DN. Podocytes are sensitive to mechanical force, implying that mechanical stretching could decrease podocyte numbers, reduce proliferation rates [56], and induce podocyte apoptosis, as well as detachment from the GBM [57]. It has been reported that podocyte numbers decrease in T1D and T2D [58] and since they have a limited capacity to renew themselves, the loss of podocytes caused by diabetes has been hypothesized to require the remaining cells to cover a larger area of GBM. Several studies in rats show that reduced nephron numbers lead to glomerular hypertrophy, podocyte injury, the

development of proteinuria, foot process widening, and subsequently glomerular sclerosis[58, 59].

1.3.3.1.1 Advanced Glycosylation End Products (AGEs)

Under normal conditions, glucose binds to proteins by a non-enzymatic chemical reaction between its aldehyde and the reactive amino groups in proteins, a reaction named the Maillard reaction [60]. Hyperglycemia increases the non-enzymatic glycation of proteins, lipids, and nucleic acid. These glycated products undergo progressive dehydration, cyclization, and rearrangement to form AGEs [60]. Once AGEs are formed, the reaction is irreversible and the proteins gradually accumulate in the tissue [61]. These advanced products can be involved in the pathogenesis of DN by altering the signal transduction via alteration in the level of soluble signals, such as cytokines, hormones and free radicals [62]. For example, it is reported that glycated albumin products stimulate type IV collagen production and inhibit proliferation in cultured mesangial cells [63]. Among the many potential pathogenic factors responsible for the development of diabetic microvascular disease, the advanced glycation pathway is thought to be a pivotal process in mediating tissue damage. The kidney contributes to increasing the circulation of AGE concentrations through dysfunctional AGE clearance. Studies in diabetic patients show that the level of AGEs are significantly increased with the progression to microalbuminuria, and subsequently, to overt nephropathy [64].

AGEs exert their action through the formation of protein cross-links altering the structure and function of the extracellular matrix (ECM), in addition to interacting with specific cell surface receptors [65]. The diverse action of AGE occurs mainly through the multi-ligand receptor RAGE, a member of the immunoglobulin superfamily of cell surface molecules [66]. AGE binding sites were identified in proximal tubules of the rat kidney, but

it is unclear whether they represent one of the known AGE receptors [67]. Numerous studies have implicated RAGEs in the development and progression of DN [67]. Yamamoto et al. reported an elevation in albuminuria, mesangial cell expansion, and advanced glomerulosclerosis in diabetic Tg mice overexpressing RAGE genes in vascular cells [68]. In contrast, the diabetic RAGE knockout mice showed less renal injury, especially in the mesangial expansion or the GBM thickening compared to the control mice [69, 70].

AGEs induce activation of ROS and upregulate inflammatory gene expression. The intracellular accumulation of AGEs initiates several signaling events by producing free radicals[67]. In turn, ROS activation initiates several intracellular pathways including PKC, mitogen-activated protein kinase (MAPK), nuclear transcription factor (NF-κB), and increases the production of cytokines, including transforming growth factor (TGF-β), interleukin (IL-6), and tumor necrosis factor (TNF-α) [60, 71]. These downstream AGE-mediate effects exacerbate renal damage. AGE signaling can be blocked in cells by expressing RAGE antisense cDNA or anti-RAGE ribozyme [72]. Furthermore, administration of aminoguanidine (AGN), an AGE inhibitor, attenuates renal AGE accumulation and reduces both albuminuria and mesangial expansion [73].

1.3.3.1.2 Protein Kinase C

Hyperglycemia has been implicated in the pathogenesis of diabetic complications through the activation of the protein kinase C (PKC) system [72, 74]. PKC is a family of at least 13 isoforms of serine and threonine kinases [75]. The various PKC isoenzymes have been subdivided into three classes based on both sequence homology and mechanism of activation: 1) conventional or classical PKCs (cPKCs: PKC-α, PKC-βI, PKC-βII, PKC -γ), which are Ca^{2+} - sensitive/or dependant and activated

by both phosphatidylserine (PS) and the second messenger diacylglycerol (DAG); 2) novel PKCs (nPKCs: PKC-δ, PKC-ε, PKC-η and PKC-θ), which are Ca^{2+} - independent and regulated by PS and DAG; 3) atypical PKCs (aPKCs: PKC-ζ and PKC-λ/ι) which are Ca^{2+} - independent and do not require DAG for activation although PS regulate activation [76].

Among various signaling kinases, PKC seems to be a centerpiece in the pathogenesis of DN [70]. PKC-α and PKC-ε expression were increased in glomeruli and renal tubules of STZ diabetic rats, whereasPKC-ζ was decreased in the kidney and heart tissues compared to control [77]. PKC-β contributes to hyperglycemia-induced renal matrix production, whereby PKC- α is involved in the development of albuminuria. The expression of PKC-β was examined in human patients with diabetic nephropathy. PKC-β mRNA was increased in the kidney biopsies of diabetic patients as compared to control subjects [78]. Kelly et al. [79]reported that in vivo inhibition of the PKC- β isoform with Ruboxistaurin, in STZ-induced diabetic rats, led to reduction in renal TGF- β 1 expression and structural injury of the kidney, as well as albuminuria. Similarly, in type 2 diabetes, Ruboxistaurin mesylatealso reduces renal abnormalities in db/db mice suggesting a central signaling role in hyperglycemia-induced vascular injury [80]. Furthermore, Chan's group have reported that PKC-βI activation induces osteopontin mRNA expression in IRPTCs [81]. Manne et al.demonstrated that STZ-induced diabetic PKC-α $^{-}/^{-}$ mice are protected against the development of albuminuria, whereas increased TGF β-1and renal hypertrophy are not prevented [82]. Matthias et al. demonstrated that PKC-β-deficiency (PKC-β $^{-}/^{-}$) in vivo reduces renal hypertrophy but not albuminuria in the STZ-induced diabetic mouse [83]. Dual inhibition of PKC-α and PKC-β isoforms (homozygous PKC-α/β double knockout) decreased in glomerular hypertrophy, extracellular

matrix and TGF-b when compared with wild-type mice after 8 weeks of diabetes [84].

In diabetic conditions, the cellular events of polyols, the generation of AGE products, and ROS activate PKC [72]. It has been shown that hydrogen peroxide (H_2O_2)can activate PKC either directly or by increasing DAG production [85]. The potential mechanism for PKC activation by ROS is through redox changes in sulfhydryl groups on PKC isoform cysteine-rich regions. These redox changes may also cause PKC isozymes to be more responsive to DAG activation during signal transduction [86]. Experimental studies show that the coactivation of PKC and MAPK in the presence of high glucose concentrations suggests that these two families of enzymes are linked [87].

1.3.3.1.3 Increased Polyol Pathway Flux

The polyol pathway is one of the glucose metabolic pathways that plays a significant role in the pathogenesis of diabetic complications [55]. Glucose use, in the polyol pathway, is 3% in normoglycemic individuals and 30% in hyperglycemic individuals [88]. In this pathway, two enzymes are involved: aldose reductase (AR), which plays the central role, and sorbitol dehydrogenase (SDH). AR normally reduces toxic aldehydes in cells to inactive alcohols. In the first enzymatic reaction, AR reduces glucose to sorbitol using NADPH as a cofactor. It is important to mention that sorbitol plays a role in balancing the osmotic pressure of extracellular NaCl to prevent cellular dehydration [88]. In the second enzymatic reaction, SDH with its co-factor NAD^+ converts sorbitol to fructose [89].

When intracellular glucose becomes elevated in the kidney, it will cause glucose flux through the polyol pathway leading to a marked increase in aldose reductase activity, with accumulation of sorbitol altering the $NADPH/NADP^+$ ratio [72]. NADPH is essential for regenerating critical

intracellular antioxidants. Sorbitol production depletes NADPH, consequently reducing glutathione (GSH) levels of nitric oxide (NO) production by the endothelial cells, which increases the susceptibility to intracellular oxidative stress and alters the antioxidant balance [90]. This is in agreement with the Lee et al. study, in which a decreased level of GSH was found in the lenses of AR-Tg mice [91]. In the kidney, studies have shown that AR protein was localized to podocytes and distal convoluted tubules [92]. Type 1 diabetes is associated with increased renal sorbitol and fructose levels [93]. Type 2 diabetic patients had higher serum and urine myo-inositol concentrations and sorbitol excretion than healthy controls [94].In streptozotocin[95]diabetic rats, sorbitol accumulation has been found in isolated glomeruli. The consequences of heightened sorbitol pathway activity include non-enzymatic glycation initiated by fructose, a glycating agent that is ten times more potent than glucose in activating PKC [96]. Theories proposed for the pathogenesis of diabetic nephropathy are illustrated in figure 1-6.

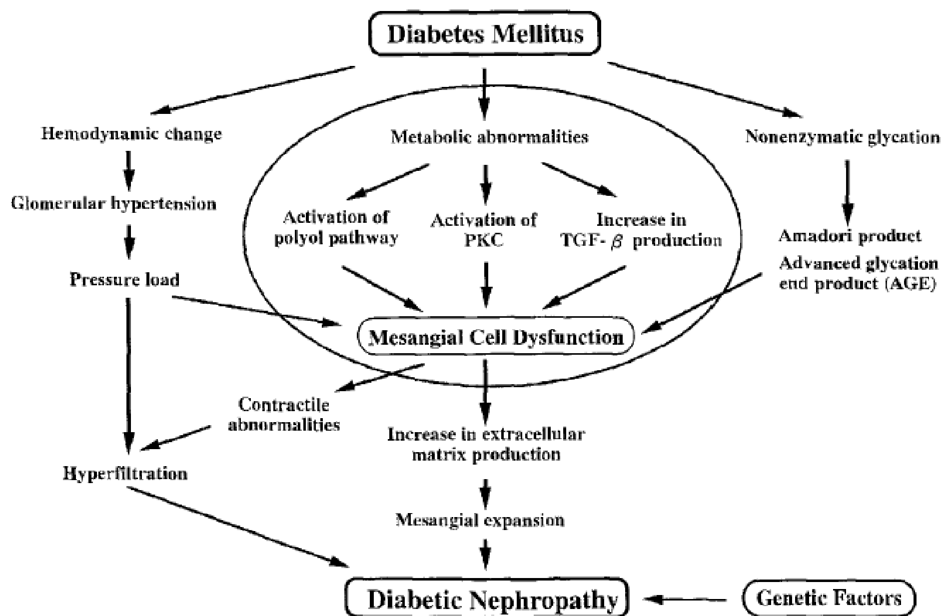


Figure 1-6: Proposed mechanisms for the pathogenesis of diabetic nephropathy [60].

1.4 Insulin

Insulin is a peptide hormone discovered in the 1920s. This hormone is secreted by the Beta cells of the pancreatic islets of Langerhans in response to increased circulating glucose levels after a meal [97, 98]. Insulin is implicated in a wide spectrum of biological responses, including blood glucose control and energy metabolism. It acts by stimulating glucose influx and metabolism in adipocytes and muscles, and by inhibiting gluconeogenesis in the liver. Moreover, insulin regulates the transcription of a number of genes and modulates cellular growth and differentiation [99, 100]. Since the discovery of insulin, it has been the subject of extensive research to elucidate its activity in metabolism, gene regulation, protein synthesis, and protein degradation. Impaired insulin action either due to insulin hyposecretion or defects in insulin signalling causes serious problems in glucose homeostasis and subsequently leads to DN [101].

Insulin exerts its action through binding to a transmembrane receptorthat belongs to the large class of tyrosine kinase receptors. Insulin receptors, IGF-1 receptors, and insulin receptor-related (IRR) receptors are members of the insulin receptor family and are structurally related [102, 103]. These members share more than 80% of amino acid sequence identity in the transmembrane domain with lower similarity in the extracellular domain, depending on their specific ligand [103]. The IR consists of two extracellular alpha-subunit and two transmembrane beta-subunit domains linked by disulfide bonds. Insulin bound to the extracellular domain results in receptor autophosphorylation on tyrosine residues [104]. This leads to conformational changes and enhances intrinsic protein tyrosine kinase activities of the transmembrane domain by multi-site tyrosine phosphorylation. The activated IR results in phosphorylation of several cytosolic IR substrates, such as insulin receptor substrates (IRSs), Src homology collagen (Shc) [105], and

adaptor protein with pleckstrin homology (APS), Src homology2 (SH2) domains, and Casitas B-lineage lymphoma [106]. The phosphorylated proteins dock downstream effector molecules that contain the SH2 domain, which are then able to activate two key signaling pathways. These pathways are the phosphoatidylinositol 3-kinase (PI3K)-AKT pathway that is responsible for most of the metabolic actions of insulin, and the mitogenic signaling pathway or the Ras/MAP kinase cascade, which regulates the expression of some genes and cooperates with the PI3K pathway to control cell growth and differentiation [107, 108].Figure 1-7 shows the intracellular insulin signaling transduction pathway.

1.4.1 Phosphatidylinositol-3 kinase (PI-3 Kinase) pathway

The phosphorylated IRSs mediate insulin signaling to downstream enzymes by binding to a number of proteins containing the SH2 domain. PI3K is one of the intermediate molecules that promote the insulin signal. Upon activation of PI3K by IRS, PI3K phosphorylates the phosphatidyl inositol lipids in the plasma membrane. Consequently, generated phosphatidyl inositol-3, 4, 5-trisphosphate (PIP3) recruits 3'-phosphoinositide-dependant kinase1 (PDK1). The activated PDK1 interacts with Akt. The serine/threonine protein kinases PDK1 and PKB/Akt to the plasma membrane [109]. It has been shown that AKT regulates the expression of several proteins, including glycogen synthase kinase-3 (GSK3), Glut4, NOS, and p70 s6 kinase involved in glycogen synthesis, glucose transport, vasodilation and protein synthesis, respectively. In adipose tissue PI3K appears to be important for stimulation of Glut4 to enhance glucose transport in adipose tissue [110, 111].

1.4.2 The mitogenic signaling pathway or the Ras/MAP kinase Cascade.

The activation of the MAP kinase pathway occurs when the phosphorylated Shc ctivates the growth factor receptor binder-2 (Grb2), which leads to the formation of complexes with the exchange factor mammalian son (mSOS) [112]. Grb2 can be activated by either IRS or Shc. The Grb2-mSOS complex results in subsequent activation of a series of effectors, such as Ras, Raf, MEK and the extracellular signal-regulated kinase (ERK) pathways. It has been shown that Ras is a potent activator of the MAP kinase pathway. The MAP kinase pathway involves the chronological activation of three kinases: Map kinase kinase kinase (Mapkkk), Map kinase kinase (Mapkk), and Map kinase (Mapk) [113]. Activated ERK1/2 phosphorylates a downstream ribosomal protein kinase, p90 rsk. Both ERK1/2 and p90rsk can be translocated to the nucleus where they phosphorylate translocation factors contributing to the mitogenic and growth-promoting effects of insulin. This signaling pathway is mainly implicated in cell growth, survival and differentiation [111, 114]. Zhang et al.demonstrate that insulin prevents the stimulatory effect of high levels of glucose on the expression of the renal ANG gene in IRPTC, at least in part, via the MAPK kinase signal transduction pathway, subsequently inhibiting the activation of the local renal renin-angiotensin system [115].

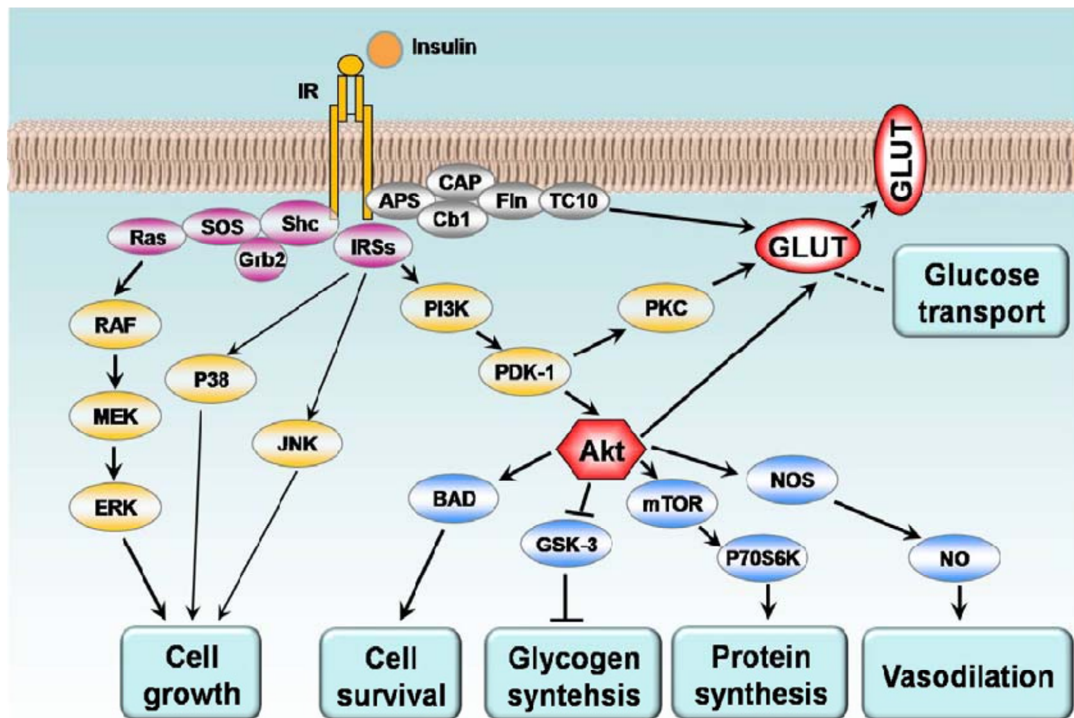


Figure 1-7. Normal intracellular insulin signaling transduction pathway.Akt, protein kinase B; APS, adaptor protein with pleckstrin homology and src homology 2 domains; CAP,Cbl/Cbl-associated protein; Cbl, Cbl family of adapters, which comprises c-Cbl, Cbl-b, and Cbl-c/Cbl-3, is implicated in receptor tyrosine kinase signaling; ERK, extracellular signal-regulated kinases; Fln, flotillin; Grb2, growth factor receptor binding protein 2; GSK-3, glycogen synthase kinase 3; IR, insulin receptor; IRS1, insulin receptor substrate 1; IRS2, insulin receptor substrate 2; JNK, Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; mTOR, mammaliantarget of rapamycin; NO, nitric oxide; NOS, nitric oxide synthase; P70S6K, ribosomal p70 S6 kinase; PCIs, percutaneous coronary interventions; PDK1, phosphoinositidedependent kinase-1; PI3K, phosphatidylinositol 30-kinase; PKC, protein kinase C; RAF, v-raf-1 murine leukemia viral oncogene homolog 1, raf proto-oncogene serine/ threonine protein kinase; Ras, a small GTP binding protein; Shc, Src homology domain adaptor homolog family member; SOS, mammalian son of sevenless; TC10, Rho familymember GTPase. [111].

1.5 The renin-angiotensin system

The renin-angiotensin system (RAS) had been previously thought of as a hormonal circulating system. Now, RAS is accepted as the body's most powerful hormone system that regulates sodium balance, body fluid homeostasis, and arterial pressure [116]. Research on the physiology of blood pressure control was initiated in 1898 by the discovery of renin, a hypertensive agent in extracts of rabbit kidney. In 1934, Goldblatt et al. demonstrated that constricting the renal arteries results in persistent hypertension in dogs due to a reduction in the vascular area with a consequent increase in strength and blood pressure [117, 118]. Later on Goldblatt confirmed that the renin was acting via a plasma substrate. The true pressor material is known as angiotensin and its precursor is angiotensinogen [117]. The historical context of discoveries related to RAS is detailed in Table 1-3.

1898	Tigerstedt and Berman discover that an extract from rabbit kidney cortex increases blood pressure
1934	Goldblatt induces hypertension in dogs by renal artery constriction.
1939–1940	Braun-Menendez et al. and Page et al. discover hypertension and angiotonin (later known as angiotensin).
1954	Skeggs et al. purify angiotensin and discover that it exists in two forms, angiotensin I and II, and predict the presence of ACE.
1954	Skeggs et al. purify angiotensin and discover that it exists in two forms, angiotensin
1954	Elliot and Peart and Skeggs et al. Report amino acid sequence of angiotensin II.
1956	Skeggs partially purifies renin substrate (angiotensinogen) and its N-terminal part.
1957	Bumpus et al. and Rittel et al. synthesize. Angiotensin II, which is widely distributed to researchers by Ciba Pharmaceuticals.
1958	Gross articulates the hypothetical relationship between angiotensin II and aldosterone.
1959–1961	Davis et al., Laragh et al. , Genest et al. , and Ganong and Mulrow demonstrate that angiotensin II regulates aldosterone secretion.
1968	Bradykinin-potentiating factor, described in 1965 by Ferreira is shown by Bahkle to inhibit the conversion of AI to AII.
1969	Radioimmunoassay methods for renin are introduced.
1969–1971	Peptide antagonists of angiotensin II are developed.
1970	Angiotensin receptors identified in vitro by Lin and Goodfriend.
1971	Elucidation of structure and synthesis of bradykinin-potentiating peptide (Teprotide).
1977	Ondetti, Rubin and Cushman describe a novel class of orally active ACE-inhibitors (captopril).
1982–1988	Orally active, non-peptide blockers of the AII type I receptor are described.

Table 1-3 shows the milestones of renin–angiotensin system history [117].

1.5.1 Renin and Pro-Renin

Renin, also known as angiotensinogenase, is an aspartyl protease that cleaves angiotensinogen into angiotensin I, the rate-limiting reaction in the cascade generating different angiotensins. In the kidney,the

juxtaglomerular apparatus (JGA) is the major site of renin synthesis [119].The active form of renin contains 340 amino acids [120]. Renin is produced from several proteolytic stages of pre-prorenin. The 406 amino acid of pre-prorenin enters the endoplasmic reticulum (ER) [121] in which the N-terminal segment (the pre-) that acts as a signal peptide is cleaved, yielding prorenin. Prorenin enters the Golgi apparatus resulting in prorenin granules that are either secreted or fused to form larger secretory granules. Consequently, as these granules mature, active renin gets glycosylated and released by exocytosis [122]. Besides the kidney (glomeruli, tubules, and vessels), adrenal glands, ovaries, testis, placenta, and retina also produce prorenin [122, 123]. Prorenin was considered to be an inactive pro-hormone that acted as an inactive precursor of renin [124]. It is now thought that prorenin may have its own activity or is converted to renin in the circulation or local tissue. There is evidence to support the special activity of prorenin [125]. There is a correlation between circulatory renin and prorenin concentration. In DN, prorenin concentration may increase in an isolated manner [126, 127]. In the kidney, it has been suggested that prorenin uptake and intrarenal activation of the kidney RAS is responsible for inducing renal damage and microvascular changes [128].

1.5.1.1 Prorenin receptor

The (pro)renin receptor ((P)RR) is a single transmembrane protein, with 350–amino acid residue, that binds prorenin and renin [129]. (P)RR is abundant in the heart, brain and placenta with lower levels being found in kidney and liver [130] as well as lesser expression in the visceral and subcutaneous adipose tissue[131]. (P)RR is a highly conserved protein in humans, rats, and mice.The binding of prorenin and renin to (P)RR is of pivotal importance with regard to the physiology of the local RAS, since it provides a mechanism to generate ANG II in a local tissue in addition to the ANG II circulating in plasma. Moreover, the binding of prorenin

induces intracellular signaling and the activation of the mitogen-activated protein (MAP) kinases ERK1/2, leading to upregulation of TGF-β1, collagen 1, and fibronectinindependent of angiotensin (Ang) II generation. Targeting of the renin receptor mRNA with siRNA blocked ERK activation and induction of TGF-beta1 [132, 133]. Additionally, (P)RR full-length cleavage by furin at a single site results in the production of a soluble form of the receptor, which is detectable in plasma. Soluble (P)RR is hypothesized to bind to specific ligands and receptors and mediate signal transduction pathways. Understanding the physiological function of full-length and soluble (P)RR will be important for establishing its role in pathology [134]. Experimental studies shows that over-expression of prorenin receptor in rats with normal renin levels may cause an increase in blood pressure, plasma aldosterone level, and promote the development of glomerulosclerosis [135]. The enzymatic cascade of the RAS, its compounds and principal functions are shown in Figure 1-8.

1.5.2 Angiotensin converting enzyme (ACE)

ACE, or kininase II is an enzyme with a wide pattern of expression and distribution in different tissues, including the lung, brain, kidney, testis, and endothelial cells of arteries and veins [136, 137]. The cloning of ACE showed that it is composed of 2 homologous catalytic domains [138]. ACE is known to be a key component of the renin-angiotensin system that regulates blood pressure. ACE functions primarily as a “peptidyl dipeptidase” that cleaves two amino acids off the C-terminus of its substrate. Its primary substrate was identified as Ang I [139]. Moreover, ACE cleaves the C-terminal of bradykinin and a number of other small peptides that lack a proline residue [140]. Bradykinin promotes vasodilation by stimulating the production of nitric oxide and arachidonic acid metabolites in the vascular endothelium. ACE determines the production of Ang II and the degradation of Ang 1–7 [141]. Therefore ACE is considered to regulate the balance between the RAS and the

Kallikrein-Kinin system [142]. In the kidney, ACE expression is located on the brush border membranes of the tubular epithelial cells [137]. ACE inhibitors (ACEi) such as Captopril and Lisinopril are used as antihypertensive agents and they act by increasing bradykinin and reducing Angiotensin II levels leading to lower blood pressure [143]. It has been shown that the ACE2 is the negative regulator that counterbalances the multiple functions of ACE in the kidneys [139]. The use of ACE inhibitors does not affect the activity of ACE2 [144].

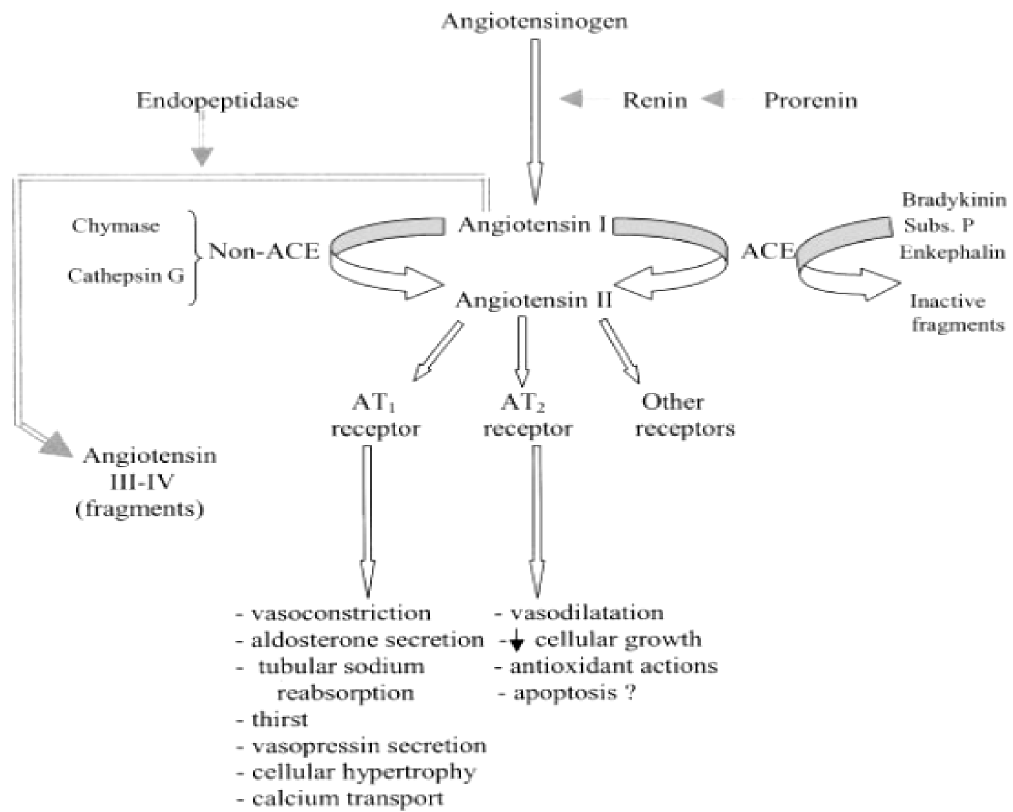


Figure 1-8: Enzymatic proteic cascade of the RAS and principal functions. [137].

1.5.3 Angiotensin converting enzyme 2 (ACE2)

ACE2 represents a zinc metalloprotease with carboxypeptidase activity [144]. It is one of the homologs of ACE. ACE2 was mapped to chromosome X [145]. ACE2 shares about 42% amino acid identities with

the catalytic domain of somatic ACE [146]. In contrast to ACE, ACE2 cleaves a single amino acid from its substrate at the C-terminal. The substrates for ACE2 are Ang I (forming Ang 1-9, from which ACE may form Ang 1-7), Ang II (forming Ang 1-7,) and bradykinin (1-8, forming BK 1-7, part of the bradykinin degradation process). The major effects of ACE2 is to convert Ang II to Ang 1-7, which binds to its own receptor, the MAS receptor [147]. In animal studies, Ang 1–7 has been proposed to play a significant role in controlling vasodilation, apoptosis, and growth arrest in the kidney [148]. The local activity of the enzyme determines the relative levels of the vasoconstrictory and pro-oxidative peptide Ang II and its vasodilatory and antioxidative metabolite Ang 1–7 at their receptors [144]. ACE2 was initially found to be expressed in endothelia of the heart and in tubular epithelial cells of the kidney [145]. In the kidneys ACE2 is notably abundant in the glomeruli, proximal and distal tubules and at the apical surface of epithelial cells [149]. ACE2 is of pivotal importance for the physiological effects of the RAS in each tissue. It has been shown that ACE2 functions as a negative regulator of the RAS and counterbalances ACE effect [139]. ACE2 could be a possible therapeutic target for disorders characterized by sodium and fluid retention like hypertension and congestive heart failure [139]. ACE2 is involved in the production of different angiotensin peptides. ACE2 function is not limited to the RAS system. In-vitro assays shows that ACE2 can remove the C-terminal residue from apelin and other vasoactive peptides such as neurotensin, kinetensin (a neurotensin related peptide), and des-Arg Bradykinin [139]. Studies have shown that *ACE2* mutant mice exhibit late-onset glomerulosclerosis and renal protein leakage [150].

1.5.4 Angiotensinogen (Agt) and angiotensins

Liver is the main source of Agt synthesis [151]. High glucose stimulates *Agt* gene expression via ROS generation in rat kidney proximal tubular cells [152]. Moreover, intrarenal RAS activation and high glucose may act

in concert to increase tubular apoptosis in diabetes [16]. The blockade of both ROS generation and activation of the intrarenal RAS improves theinhibitory action of insulin on *Agt* gene expression in IRPTCs in conditions of high glucose [153]. Recently, several studies have shown that *Agt* is the main source of different forms of angiotensin. The nomenclature for the different angiotensins is based on two systems, one of them uses the roman numeral and the other is based on the amino acid sequence that composes angiotensin I [154].

The different forms of angiotensins is listed in Table 1-4.

Substrate	Amino acid sequence	Enzyme	Receptor
Angiotensin I / (1-10)	1 2 3 4 5 6 7 8 9 10 NH2-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-COOH	ACE / ACE2/NEP	NON
Ang (1-9)	1 2 3 4 5 6 7 8 9 NH2-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-COOH	NEP and ACE	NON
Ang II /(1-8)	1 2 3 4 5 6 7 8 NH2-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-COOH	AMPA or ACE2	AT1 and AT2
Ang (1-7)	1 2 3 4 5 6 7 NH2-Asp-Arg-Val-Tyr-Ile-His-Pro-COOH	NON	MAS
Ang III / (2-8)	2 3 4 5 6 7 8 NH2-Arg-Val-Tyr-Ile-His-Pro-Phe-COOH	AMPM	AT1 and AT2
Ang IV / (3-8)	3 4 5 6 7 8 NH2-Val-Tyr-Ile-His-Pro-Phe-COOH	NON	AT4 and IRAP

Table 1-4: The amino acid sequences of angiotensin peptides (substrate), and enzymes that convert the substrate into another angiotensin [124, 154]

Angiotensins are locally produced in different organs or tissue based on activation of the RAS pathway. The angiotensin II (ANG II), angiotensin IV and angiotensin 1-7 peptides are produced upon RAS activation and interact with different cell surface receptors to induce their effects. Ang II is a multifunctional hormone that participates in multiple biological processes. Most of these actions either support or increase arterial blood pressure and maintain glomerular filtration [117]. In mammals, there are two different types of Ang II receptors, Ang II type 1 receptor (AT1R) and

Ang II type 2 receptor (AT2R). It has been shown that angiotensin IV binds to the AT4 receptor and Ang 1-7 interacts with the MAS receptor [155]. Figure 1-9 shows the different components of RAS.

1.5.5 Angiotensins receptors

1.5.5.1 Ang II type 1 receptor (AT1R)

AT1R is a polypeptide containing approximately 360 amino acids that spans the cell membrane seven times. The sequence homology between AT1R and AT2R is about 30%. In humans, AT1R is located on chromosome 3 [156]. By interacting with Ang II, AT1 receptors induce cellular responses via signaling transduction, regulating the expression of Ang II itself [157],vasoconstriction, sodium retention, and water retention. The AT1R has two isoforms, $AT1_A$ and $AT1_B$, which were identified by using specific antagonists for the angiotensin II receptor [158]. The nucleic and amino acid similarity between the two subtypes is greater than 90 [155]. In mice, the two isoforms of the AT1 receptor ($AT1_A$ and $AT1_B$) are products of differentially expressed and regulated genes [159]. The differences between the $AT1_A$, and $AT1_B$ receptor can be summarized as follows: (a) the protein sequence in the carboxy terminal tail of the molecule is different [160]; (b) there are two additional putative protein kinase C phosphorylation sites and an absence of apossible palmitoylation site in the $AT1_B$ sequence [161];(c) there is low homology (35%) in the 5' and 3' untranslated regions of the two mRNAs; and (d)the restriction maps of the $AT1_A$ and $AT1_B$ genes are quite different [161, 162]. These differences raise the possibility that there is differential regulation of these two subtypes. All of the identified clinical effects of Ang II are mediated by the AT1R. AT1Rs have been found to be expressed in the heart, kidney, brain, vascular smooth-muscle cells, adrenal glands, platelets, and in the placenta adipocytes [163, 164]. The effects of Ang II, such as vasoconstriction, aldosterone and vasopressin release, sodium and water retention and sympathetic facilitation, are all

mediated by the AT_1R. In addition, Ang II is involved in cell proliferation, left ventricular hypertrophy, nephrosclerosis, vascular media hypertrophy, and endothelial dysfunction [165]. Chan's group demonstates that RAS blockade decreases blood pressure and proteinuria in transgenic mice overexpressing rAgt gene in the kidney [166] .Moreover, dual RAS blockade normalizes *ACE2* gene expression and prevents hypertension and tubular apoptosis in Akita angiotensinogen-transgenic mice [167].

1.5.5.2 Angiotensin II type 2 receptor (AT2R)

The AT2R belongs to the superfamily of G-protein coupled receptors that contain seven transmembrane regions [168]. The AT2R is expressed abundantly during fetal development, decreasing in the postnatal period [169]. In adults the AT2R is expressed at low levels in the adrenals, pancreas, uterus, heart, vascular endothelium, and kidneys [170, 171]. AT2R is highly conserved across species and tissues within species. The nucleic acid sequence homology between rat and human AT2R is around 90% [168, 172]. The physiological role of the AT2R is unclear and only partly understood. It has been shown that AT2R function counterbalances some of the effects of Ang II mediated by AT1 receptors. AT2R stimulation results in an antiproliferative effect/inhibition of cell growth, cell differentiation, tissue repair, vasodilation, and kidney and urinary-tract development [173]. Malfunction of the AT2R gene seems to contribute to congenital anomalies of the kidney and urinary tract [173].

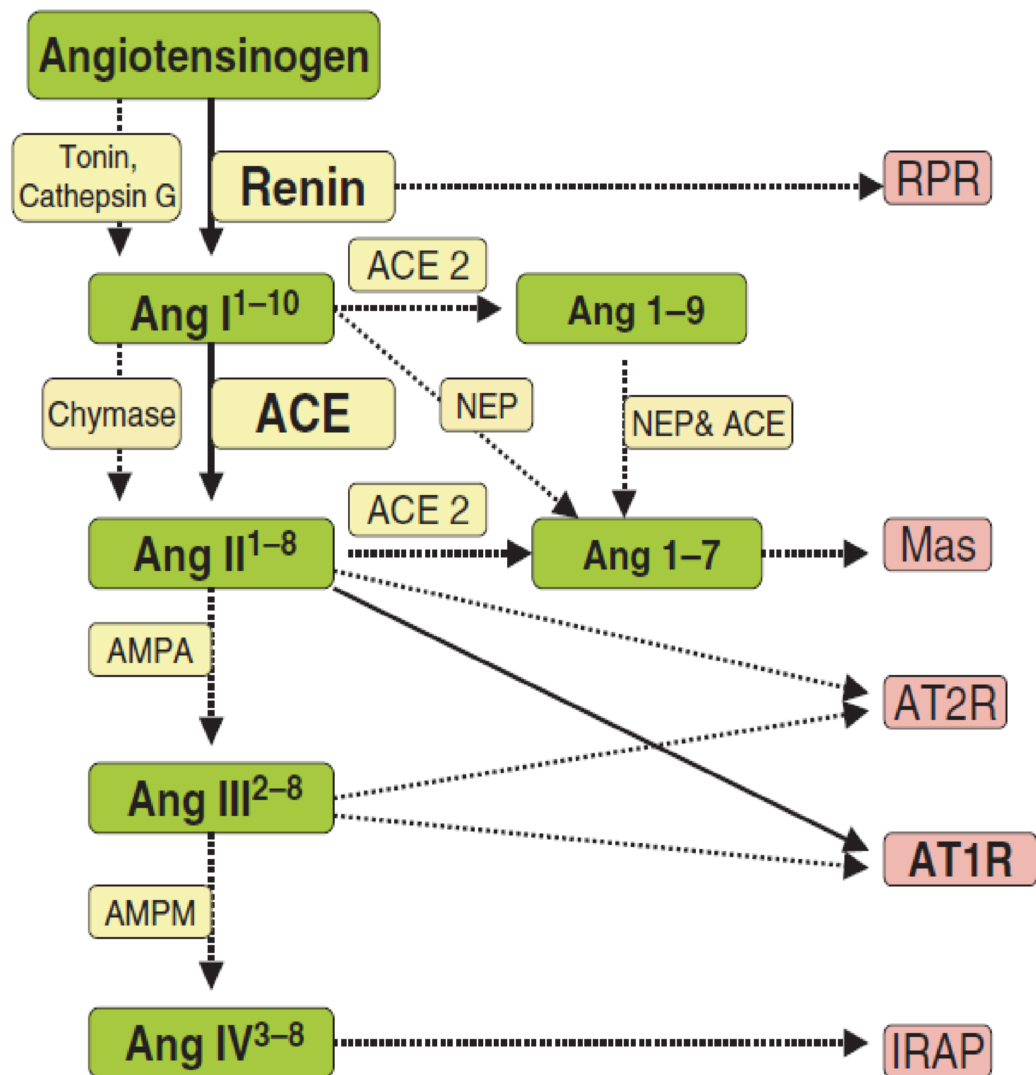
1.5.5.3 AT4 receptor (AT4R)

Angiotensin IV has been demonstrated to acquire unique pharmacological properties that are independent of classical angiotensin receptors (AT1R and AT2R). Angiotensin IV may be generated from Ang III by aminopeptidase M [174]. In 1992, the first study on the angiotensin AT4R showed that AT4R had a specific and high-affinity binding site for the hexapeptide angiotensin IV (Ang IV) [175]. Structure activity studies

revealed that the first three amino acid residues of Ang IV are critical for binding to the AT4R[176]. AT4R has been detected in spleen, colon, prostate, bladder, and the kidney [177]. The AT4R was reported to occur at high levels in the proximal tubules [178].

1.5.5.4 Mas oncogene receptor (MAS)

As mentioned above, ACE2 is able to hydrolyze Ang I into Ang-(1-9), which is converted to Ang-(1-7) via ACE. Moreover, ACE2 more efficiently catalyzes the conversion of Ang II to Ang-(1-7) making Ang II the major substrate for Ang-(1-7) synthesis [179]. Studies using the selective Ang-(1–7) antagonist A-779 [180] support the existence of an Ang-(1–7) receptor distinct from the classical Ang II receptors AT1R and AT2R [180, 181]. The study by Santos et al. provided the first evidence for a functional role of the *Mas* receptor as the mediator of the Ang-(1–7) effects in thevascular system [182]. The Masproto-oncogene was first detected through its tumorigenic activity in *in vivo* tumor assays [183]. The *Mas* receptor was first cloned and sequenced in NIH 3T3 cells from nude mice by Young et al. [184]. It encodes a very hydrophobic protein with 7 transmembrane domains featuring characteristics of class I G-protein–coupled receptors [185]. In mammals, the gene is expressed predominantly in the testis, brain, and in detectable levels in the kidneys and heart [183, 186]. The protective effect of Ang-(1-7) has been shown in animal models of kidney disease as its administration reduced the amount of urinary protein excretion [187]. ACEi and ARB cause increases in *ACE2* gene expression and plasma Ang-(1-7). Therefore, the *ACE2*/Ang-(1-7)/*Mas* axis could be a potential therapeutic target to control blood pressure [148, 188]. The present view of the expanded renin-angiotensin systemis is shown in figure 1-9.



Figue 1-9: The present view of the expanded renin-angiotensin system. RPR, renin⁄prorenin receptor; *Mas*, *mas* oncogene, receptor for Ang-(1–7); AT2R, angiotensin type 2 receptor; AT1R, angiotensin type 1 receptor, IRAP, insulin-regulated aminopeptidase; Ang IV receptor AMPA, aminopeptidase A; AMPM, aminopeptidase M; *ACE*, angiotensin-converting enzyme; *ACE2*, angiotensin-converting enzyme 2; NEP, neutral endopeptidase [147].

1.6 Reactive oxygen species

The Reactive Oxygen Species (ROS) includes a number of reactive molecules and free radicals derived from molecular oxygen. Although oxygen is vital for aerobic respiration, about 5% or more of the inhaled O_2 is transformed to ROS such as O^-_2, H_2O_2, and •OH by the univalent reduction of O_2[189]. Living organisms produce ROS as a by-product of normal cellular metabolism. ROS are produced in all cell types and serve as important cellular messengers for both intra- and inter-cellular communications. Physiological levels of ROS are beneficial for the cells and at normal physiological conditions; ROS activate the transcription of vascular genes and act on the immune system as effectors molecules against pathogens. Moreover, ROS are a key messenger in signal transduction and cell cycling under normal cellular conditions [190]. The cell's response to ROS depends on the intensity, duration, and context of the signaling induced but high levels of ROS, however, produce adverse modifications to the cell components, such as lipids, proteins, and DNA. ROS can activate stress reponse genes [191].

1.6.1 Endogenous Sources of ROS

Normally, different free radical species are produced in the body to carry out particular functions. Free radicals are chemical species containing a single unpaired electron, which is highly reactive as it seeks to pair with a new free electron to form either another free radical or a paired electron — ultimately leading to damage to the cellular and tissue system [192, 193]. Superoxide (O_2^{-}), hydrogen peroxide (H_2O_2), and nitric oxide (NO) are free radical reactive oxygen species (ROS) that are essential for normal physiology, as well as mediating cellular degeneration in disease states [194]. Reactive oxidants include ROS (i.e. $O_2^{\bullet-}$, H_2O_2, •OH, RO_2•, RO•, $^1O_2$, and O_3) and RNS (i.e., •NO, •NO_2, and $ONOO^-$).Cellular sources of ROS production include the plasma membrane NADPH oxidase,intracellular cytosolic xanthine oxidase, peroxisomal oxidases,

endoplasmic reticular oxidases, and mitochondrial electron transport components. The mitochondria are considered the main source of ROS production from aerobic respiration under physiological conditions and are implicated in many pathophysiological diseases and disorders [195, 196]. ROS arise from the synthesis of nitric oxide (•NO) by NO synthase, which react with superoxide (O_2•−) to form a more stable compound [197]. The free radical nitric oxide (•NO) is a product of the oxidation of L-arginine to L-citrulline in a two-step process catalyzed by the enzyme nitric oxide synthase (NOS) [198]. Physiological levels of nitric oxide play a role in regulating blood pressure and may be involved in the killing of parasites by macrophages [199]. Superoxide may also be involved in the 'sensing' of blood O_2 levels by the carotid body.ROS may be divided into 2 groups: free radicals and nonradical derivatives (Table 1-5). It has been estimated that the production of H_2O_2 at nanomolar levels is required for proliferation in response to growth factors [200]. In the absence of catalyzing agents (e.g. enzymes, multivalent metals, etc.), hydrogen peroxide (H_2O_2) reacts with thiolate anion (S-) to form sulfenic acid, which in turn ionizes to form sulfenate (SO^-). This intermediate can be reversed by the action of glutathione [201].

Reactive Oxygen Species (ROS)	
Radicals	*Nonradicals*
Superoxide, O_2•$^-$	Hydrogen peroxide, H_2O_2
Hydroxyl, OH•	Hypochlorous acid, HOCl
Peroxyl, RO_2• (e.g. lipid peroxyl, see text)	Hypobromous acid, HOBr
Alkoxyl, RO•	Ozone, O_3
Hydroperoxyl HO_2•	Singlet oxygen $^1\Delta_g$
Reactive Nitrogen Species (RNS)	
Radicals	*Nonradicals*
Nitric oxide (nitrogen monoxide), NO•	Nitrous acid, HNO_2
Nitrogen dioxide, NO_2•	Nitrosyl cation, NO^+
	Nitroxyl anion, NO^-
	Dinitrogen tetroxide, N_2O_4
	Dinitrogen trioxide, N_2O_3
	Peroxynitrite, $ONOO^-$
	Peroxynitrous acid, ONOOH
	Nitronium (nitryl) cation, NO_2^+ (e.g. as nitryl chloride, NO_2Cl)
	Alkyl peroxynitrites, ROONO

Table 1-5: Shows types of radicals in oxidative stress conditions [199].

1.6.2 Oxidative stress

The term "oxidative stress" refers to a shift in balance between oxidant/antioxidant in favor of oxidants [199]. Oxidative stress is a key impairment induced by various conditions, including atherosclerosis, hypertension, acute respiratory distress syndrome, chronic obstructive pulmonary disease, asthma, hepatitis, pancreatitis, cancer, and neurodegenerative diseases [190, 202-204]. The excess production of free radicals can deplete intracellular antioxidants, resulting in oxidative stress. It has been shown that higher levels of ROS induce necrotic cell death whereas lower levels lead to apoptosis [190].

1.6.3 Consequences of oxidative stress

Oxidative stress resulting from an imbalanced ratio between ROS production and detoxification may also disturb physiological signal transduction, lead to chain reactions in lipid layers, and damage DNA repair enzymes [193]. In certain pathological conditions, increased generation of ROS and/or depletion of the antioxidant defense system leads to enhanced ROS activity and oxidative stress, resulting in tissue damage. Hypertension, diabetes mellitus, metabolic syndrome, smoking, as well as alcohol consumption induce renal OS [205]. The kidney is an organ highly vulnerable to damage caused by ROS, likely due to the abundance of long chain polyunsaturated fatty acids in the composition of renal lipids [205]. Excessive ROS production in the kidney has been reported in different hypertensive animal models, including angiotensin II-induced hypertensive rats, N-omega-nitro-L-arginine-induced hypertensive rats [206], Dahl salt-sensitive hypertensive rats [207], and spontaneously hypertensive rats [208] . Blockade of both ROS generation and activationof the intrarenal RAS improves theinhibitory action of insulin on *Agt* gene expression inIRPTCs in conditions of high glucose [153].
ROS activate to translocate into the nucleus to bind and activate the transcription of ARE-bearing genes [209].

1.6.4 Diabetic nephropathy and ROS

Chronic hyperglycemia causes oxidative stress in tissues prone to complications in patients with diabetes [191]. Hypertension is one of the major causes of the onset of renal failure. In diabetes, increased ROS is commonly found in the tissues affected by hyperglycemia, including the kidney [191, 210].

1.6.5 Antioxidant

Antioxidants are defined as any compound that can donate at least one hydrogen atom to a free radical, resulting in the termination of radical chain reactions [194]. Reactive species are regulated by a complex web of antioxidant defenses that diminish the oxidative damage to the biomolecules (see Table 1-6). It has also been suggested that antioxidant activity itself may be a consequence of ROS production [211]. Dietary antioxidant forms the first line of defense against oxidative stress. Together, water-soluble vitamin C (ascorbic acid) and fat-soluble vitamin E make up an antioxidant system in mammalian cells [212]. Antioxidant treatment attenuates renal ROS production and renal injury in the DOCA-salt hypertensive rats [213]. In this model, treatment with a NADPH oxidase inhibitor significantly reduced deoxycorticosterone (DOCA-salt) superoxide production in the aorta [214]. In the human disease, this 'oxidant–antioxidant' balance is tilted in favour of the reactive species, so that oxidative damage levels tend to increase. In the diabetic rat kidney, administration of vitamin C increases the activity of antioxidant enzymes such as catalase (CAT) and glutathione peroxidase (GSHPx) [215].

1.6.5.1 Catalase

Under physiological conditions, H_2O_2 formation and elimination are kept in balance by antioxidant enzymes. The initial step in all ROS formation is the conversion of oxygen to superoxide anion (O_2^-), which in turn is converted to the less-reactive hydrogen peroxide (H_2O_2) by superoxide

dismutases (SOD) [216]. Catalase, superoxide dismutase (SOD), and glutathione peroxidase are antioxidant enzymes produced naturally within the body [193]. Catalase is a 240 kDa homotetrameric enzyme that catalyzes the production of water and molecular oxygen from H_2O_2, thereby protecting the cell from oxidative stress damage. It is expressed mainly in the peroxisomes and to some extent in the cytosol where H_2O_2 is detoxified [217]. Increased oxidative stress is commonly found in tissues affected by diabetes and hypertension.In the diabetic rat kidney, catalase and glutathione peroxidase (GSHPx) activities are reduced [215]. The work by Chan's group has shown that overexpression of catalase prevents hypertension and tubulointerstitial fibrosis and normalization of renal angiotensin-converting enzyme-2 expression in Akita mice[218]. Similarly, catalase overexpression in RPTs of non-diabetic mice prevents hypertension and tubular apoptosis in angiotensinogen transgenic mice [219].

Enzymatic Scavenger of Antioxidant Defense

Name of Scavenger	Acronym	Catalyzed Reaction
Superoxide dismutase	SOD	$M^{(n+1)+}$-SOD + O_2^- → M^{n+}-SOD + O_2 M^{n+}-SOD + O_2^- + $2H^+$ → $M^{(n+1)+}$-SOD + H_2O_2
Catalase	CAT	$2\ H_2O_2 \rightarrow O_2 + 2\ H_2O$ H_2O_2 + Fe(III)-E → H_2O + O = Fe(IV)-E(.+) H_2O_2 + O = Fe(IV)-E(.+) → H_2O + Fe(III)-E + O_2
Glutathione peroxidase	GTPx	$2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$ 2GSH+ ROOH → GSSG + ROH + H_2O
Thioredoxin	TRX	Adenosine monophosphate + sulfite + thioredoxin disulfide = 5'-adenylyl sulfate + thioredoxin Adenosine 3',5'-bisphosphate + sulfite + thioredoxin disulfide = 3'-phosphoadenylyl sulfate + thioredoxin
Peroxiredoxin	PRX	2 R'-SH + ROOH = R'-S-S-R' + H_2O + ROH
Glutathione transferase	GST	RX + GSH = HX + R-S-GSH

Table 1-6: A group of cellular defense genes and their role in ROS neutralization [193]

1.7 Promoters

The eukaryotic gene can be divided into different regions. Gene transcription occurs strictly in the 5' -to- 3' untranslated region (UTR) direction. The gene promoter is located at the 5' UTR flanking region upstream from the start codon. At the 3'UTR flanking region, there is the stop codon and the polyadenylation site.The existence of the promoter is a prerequisite for gene transcription from DNA. The promoter is defined as the sequence of DNA upstream of the transcriptional start site (TSS) that serves to recruit the polymerase complex that will read the DNA to produce an RNA transcript [220]. In the eukaryote, the promoter region is divided into three regions. The basal promoter or core promoter region is conventionally located -35 to +35 relative to the transcription start site (TSS) [221]. Lying directly upstream from the core promoter is the proximal promoter. This region contains regulatory elements that code for gene activator ROS, which can activate stress response genes (for example, pathways triggered by too many ROS binding sites). There is also the distal promoter region, which contains distant regulatory elements [222, 223]. Other elements that control gene transcription are enhancers and silencers. Enhancers can be located upstream or downstream, or even within the gene they control, but they are frequently located upstream from the TSS [224]. Enhancers contain binding sites for transcription factors that increase the rate of gene transcription. On the other hand, silencers can inhibit transcription of the gene through different mechanisms, e.g. by interfering with activator binding and/or by preventing recruitment of the transcriptional machinery and modifying chromatin structure [224]. Some features of promoter sequences are the TATA box and CpG islands. The TATA box is a consensus of the ATATA (A/T)A(A/T) sequence [225]. The TATA box is located 25-50 bp upstream of the transcription site [226]. The TATA-binding protein (TBP) guides the RNA Pol II recognition of the start site(s) on a gene. It was found that RNA Pol II, the general transcription factors (GTFs), and the

TBP only support basal transcription and do not respond to gene-specific activators[227]. CpG islands in the promoter sequence are phosphodiesterase-linked cytosines and guanines,and appear at high frequenies in most DNA promoters [225, 227]. Chan's group identified a putative insulin-responsive element (IRE) in rat angiotensinogen gene promoter that binds with two nuclear proteins under the action of high glucose and insulin [228]. Moreover, the molecular mechanism(s) of insulin action are mediated, at least in part, via interaction of the functional IRE with *hnRNP F* (48-kDa) [229] and *hnRNP K* (70kDa) [230] nuclear proteins in the rat ANG gene and are MAPK-dependent. An example of the eukaryotic insulin gene promoter is shown in Figure 1-10.

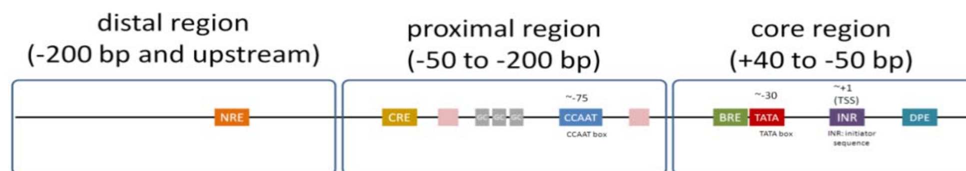


Figure 1-10: Human insulin gene promoter as an example for eukaryotic promoter [231].

1.7.1 Promoter function

A promoter is essential for transcription initiation. Promoter sequences have several regions that regulate the speed of transcription into the desired protein. If we identify the DNA segment corresponding to the promoter sequence, it will enable the understanding of how these sequences interact with various transcription factors to regulate gene expression. Using the promoter sequence in transgenic animals to look for gain-of-function or loss-of-function is a useful tool in assessing function at a promoter[232].

1.7.1.1 Promoter analysis and software

Most prediction algorithms do not provide high enough sensitivity and specificity for promoter prediction. There are numerous challenges in

accurately predicting promoter sequences as the promoter region represents a very small proportion of the entire genome. The length of a promoter varies from a few hundred bases in some genes to thousands in others. Insufficient and incomplete information about transcription factors (TFs) and TF binding sites adds to thechallenge of understanding the nature of binding to a specific binding site in the promoter sequence [220, 232]. Promoter prediction software is available online and can be a good starting point for designing reporter assays or for looking for potential motifs for TFs that might regulate a target gene of interest.

1.7.2 Transcription factors (TFs)

Expression of protein-coding genes in eukaryotes is a multistep process that starts with the initiation of transcription by RNA polymerase II at the promoters [226]. It is now well established that the primary control of gene expression lies at the level of gene transcription with many genes being transcribed in tissue and temporal-dependent contexts requiring a specific protein[232].Transcription factors are proteins that control which genes are turned on or off in the genome; they bind directly to certain DNA sequences to activate or inhibit the enzyme that controls the transcription of genes. In addition, these transcription factors can interact with each other and with the RNA polymerase enzyme itself in order to modulate transcription [220, 232]. Transcription factors are essential for the regulation of genes. They are usually members of multigene families. For example, members of the Rel family serve as central regulators of the cellular defense response against stress, injury, and external pathogens [233]; the basic leucine zipper (bZIP) transcription factors(the Cap ‘n’ Collar (CNC) family [234, 235]) interact with *Keap1* (Kelch-like ECH-associated protein 1); and the heterogeneous nuclear ribonucleoproteins family [236] plays a functional role in RNA biogenesis [237].

1.7.2.1 The heterogeneous nuclear ribonucleoproteins

The heterogeneous nuclear ribonucleoproteins (hnRNPs) are a large family of nucleic acid binding proteins. HnRNPs have been identified in the budding yeast *Saccharomyces cerevisiae* and most eukaryotes [238-240]. The HnRNPs family consists of ~30 members [241]. Subsets of hnRNPs are strictly nuclear, while the others shuttle between the nucleus and cytoplasm [241]. Some nuclear proteins, including hnRNP proteins, exit the nucleus and rapidly re-enter in a process referred to as nucleocytoplasmic shuttling [242]. The main function of the proteins that shuttle between the nucleus and the cytoplasm is to mediate RNA export [243].

1.7.2.1.1 Heterogeneous nuclear ribonucleoprotein (*hnRNP K*) characterization.

HnRNP K was first characterized as a component of the hnRNP family in 1992 [244]. K protein is encoded by a gene mapped to chromosome 9, 17 and 13 in humans, rats, and mice, respectively [245-247]. Cellular *hnRNP K* isoforms are produced as a result of alternatively spliced transcripts that vary by small insertions of five and/or 20 amino acids, respectively. SDS-PAGE shows that the molecular weight of the isoforms is in the range of 65-70 kD [248]. The predicted amino acid sequence of K does not show extensive homology to sequences of any known proteins [244]. *HnRNP K* gene contains evolutionarily conserved KH repeats that provide a structural basis for mRNA binding. The KH module is a sequence motif identified in a number of diversified RNA-binding proteins and is suggested to be the functional element responsible for RNA binding [249], as well as enabling these proteins to engage in both protein/nucleic acid and protein/protein interactions [250]. KH-like domains are found in RNA-binding proteins in species as diverse as *Escherichiacoli* and *Saccharomyces cerevisiae* [251]. KH domains are almost completely conserved between *Xenopuslaevis* and mammals [252].

The most prevalent domain amongst the hnRNPs is the RRM that mediates specific interactions with the pre-mRNA except *hnRNPs E/K* [237]. *HnRNPs E1*, *E2* and *K* contain three KH domains instead of RRMs or RRM-like domains that enable them to interact with pyrimidine rich binding sequences in target RNAs [253]. The KH domain was initially defined in *hnRNP K* as a conserved region of 45–55 amino acid residues that are repeated three times in this protein [254]. The three KH domains of *K* protein are almost completely conserved between X. Laevis and mammals, [255] and are well conserved between flies, nematodes, and yeast [256]. The structures of KH domains of various RNA-binding proteins have been studied using NMR and X-ray crystallography [257]. It has been demonstrated that KH-domains 1 and 2 of *hnRNP K* are separated from domain 3 by different motifs with the first domain containing two RGG boxes [254]. Most hnRNPs also harbor RGG boxes (repeats of Arg-Gly-Gly tripeptides) and additional glycine-rich, acidic, or proline-rich domains [253]. The NMR structure of the KH3 domain of the K protein bound to a single-stranded DNA of sequence TCCCT was resolved [257]. Dejgaard and Leffers [249] found that the poly (rC)-binding of hnRNP K is mediated by the third KH domain (KH3). Similarly, Ito et al. [258] found that hnRNP K binds dC-rich single-stranded DNA via the carboxyl terminus containing the KH3 domain. In contrast, Siomi et al. [259] suggested that all three *hnRNP K* KH domains, KH1, KH2 and KH3 play a role in binding to poly(rC) under stringent conditions (1 M NaClconcentration). The N terminus of the K protein is highly acidic and has been reported to have transcriptional activity [260]. Morover, *hnRNP K* protein contains two internal repeats not found in other known proteins, as well as Gly-Arg-Gly-Gly and Gly-Arg-Gly-Gly-Phe sequences, which occur frequently in many RNA-binding proteins [244]. K protein modular domains are illustrated in Figure 1-11.

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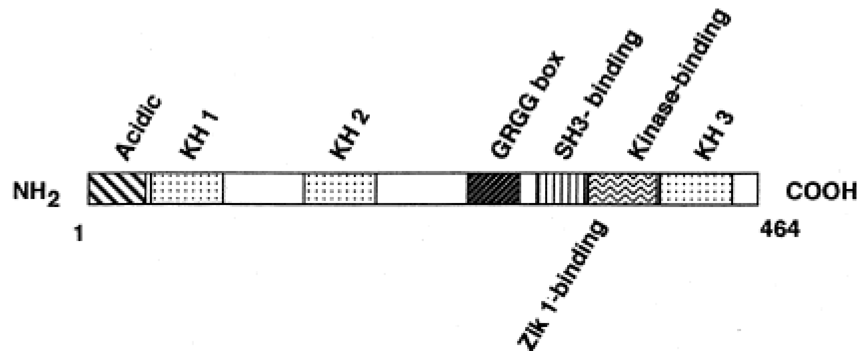

Figure 1-11: Diagrammatic illustration of K protein modular domains. Acidic domain is contained in aa 1-40; KH 1 aa 46-98; KH 2 aa 149-197; KH 3 aa 391-439; GRGG box aa 236-273; Src SH3-binding domain aa 289-315; Zikl-binding domain aa 209-337; K protein kinase (KPK)-binding domain aa 337-425 [250]

1.7.2.1.2 HnRNP K localization and function

It was assumed that all hnRNPs proteins were restricted to the nucleus [261]. However, hnRNP K protein is abundantly found in the nucleus, cytoplasm, and mitochondria belonging to a set of RNA-binding proteins, characterized by preferential binding to C-rich binding sites [244, 262]. Immunofluorescence microscopy showed that hnRNP K had been localized to the nucleus and cytoplasm [263]. It has been reported that hnRNPK shuttles between the two cellular compartments. The functional versatility of *hnRNP K* arises from its three KH domains that can interact with both RNA and ssDNA [255]. K protein interacts with a diversity of molecules involved in gene expression and signal transduction [255, 264] including RNA, DNA [262], and factors involved in chromatin remodeling [265]. HnRNP K is a nuclear protein that binds to the insulin-responsive element of the rat angiotensinogen gene promoter and modulates angiotensinogen gene transcription in the kidney[230]. Gardner's group has shown the differential effects of *hnRNP K* on Sp1 and Sp3-mediated transcriptional activation of neuronal nicotinic acetylcholine receptor

promoter [266]. In 2004 Bomsztyk et al. showed that *hnRNP K* acts as a 'docking platform' to coordinate nucleic acid metabolism and mediate the cross talk between signaling pathways. As a transcription factor *hnRNP K* may regulate gene transcription [255, 267]. For example, K protein binds the homopurine *HnRNP K* /homopyrimidine (CCCC/ GGGG) tract present in the CT motif [250], nested within the c-*myc* promoter PI [267] and the KB motif [268]. The ERK kinase phosphorylates the Serine residue of *hnRNP K* leading to accumulation in the cytoplasm, and enhances the DICE-dependant inhibition of mRNA translation [269]. This finding shows that hnRNP K is subject to both positive and negative regulation by different cellular signals [270]. Matunis et al have shown that *hnRNP K* and *J* play a role in the nuclear metabolism of hnRNAs, particularly for pre-mRNAs that contain cytidine-rich sequences [244]. *HnRNP K* and *E1/E2* function as regulators of cytoplasmic mRNAs, particularly in erythroid cells. Although *hnRNP K* and *hnRNP E1* share a common structural motif, the *hnRNP K* homology (KH) domain is the substrate for c.src phosphorylation but *hnRNP E1* is not [254].

1.7.3 Heterogeneous nuclear ribonucleoprotein F (hnRNP F)

HnRNP F belongs to the hnRNPs family and its cDNA *hnRNP F* was isolated by Matunis et al. in 1994. The purified hnRNP F protein contains 415 amino acids, with an apparent molecular weight of 50kDa on SDS-PAGE [271]. The open reading frame of rat *hnRNP F* cDNA shows a 98% similarity to the human counterpart [272]. *HnRNP F* is an abundant protein present in the thymus, spleen, and testis [271, 273]. Dominguez et al. reported on the role of the *hnRNP F* in alternative splicing regulation by remodeling RNA structures [274]. Moreover, *hnRNP F* has the ability to bind ssDNA as well as RNA, with a preference for oligo (dG) [271, 275]. Repeats of at least three consecutive guanines, known as G-tracts, have been reported to be over-represented in RNA molecules, especially near splice sites [275]. Transient transfer of sense and antisense *hnRNP*

F cDNA in IRPTCs inhibited and enhanced *Agt* gene expression, respectively. Thus dysregulation of *hnRNP F* might affect renin-angiotensin system activation and, subsequently, kidney injury in diabetes [229].

1.7.3.1 HnRNP F and other hnRNPs

Interestingly, the cellular distribution of *hnRNP K* is similar to *hnRNP F*, but the relative levels of gene expression for each of them are different [271]. Structural similarity between *hnRNP F* and other hnRNP members has been reported in several studies and *hnRNP F* and *hnRNP H* are highly similar in sequence, structure, and binding preferences [272]. In HeLa cells, *hnRNP H/F* are nuclear localized at steady state, but both proteins are localized to a certain degree in the cytoplasm in some tissues, while substantial cytoplasmic relocalization (especially for *hnRNP F*) is reported in some tumors [276]. Although *hnRNP F/H* proteins are recognized as regulators of alternative splicing [277, 278], they could function as activators of gene expression of certain genes under certain conditions [279]. Cross-linking immunoprecipitation assays show that the binding site consensus sequence for *hnRNP* is GU-rich, while *hnRNP H* is GA-rich [280]. The expression of *hnRNP F/ hnRNP H* antagonize each other in the regulation of polyadenylation of mRNAs and display different binding specificities for gene regulatory elements [281]; for instance, the differentiation of memory cells into B-cells is regulated by *hnRNP F.* The protein level of *hnRNP H* in memory cells is higher than *hnRNP F*, which may prevent *hnRNP F* from promoting the differentiation of memory cells into B cells [282]. *HnRNP F* protein overexpression, but not *hnRNP H*, increases cell proliferation, whereas the knockdown shows the reverse effect [261]. It has been reported that cell proliferation is regulated through *hnRNP F* function in the nucleus through S6K2 and mTOR. In a recent example, *hnRNP F* regulates the alternative splicing of exon 11 in the insulin receptor gene [283]. The dysregulation of hnRNP is a

contributor to disease progression such as diabetes. Work by Chan's group showed that overexpressing *hnRNP F* and *hnRNP K* leads to the inhibition of renal angiotensinogen gene expression. Moreover, many hnRNPs are involved in telomere biogenesis involved in tumorigenesis. Changes in the activities of *hnRNPs A1* and *F/H* could lead to mis-splicing of their mRNA target genes, which include cell proliferation genes. Defects in *hnRNP E/K* alternative splicing for *c*-Myc, the androgen receptor, *eIF4E* (eukaryotic translation initiation factor 4E), and *p53* will cause unnecessary cell proliferation. HnRNPs dysregulation and contribution to carcinogenesis is shown in Figure 1-12[261].

1.7.4 The CNC-bZIP family

Eukaryotic cells are frequently exposed to different environmental insults and harmful substances such as ROS as a side product of metabolic reactions. Cells have developed adaptive mechanisms to counteract the environmental stresses referred to as the detoxification process. This metabolic process consists of three phases (I, II, and III) [284, 285]. In phase I, the family of cytochrome P-450 enzymes is responsible for adding a functional polar group to the xenobiotic insult. Phase II further inactivates the electrophilic metabolites while in phase III the inactive metabolites are expelled outside the cell by the action of the membrane transporters of the multidrug resistance protein family [286, 287]. One of the gene families that play a role in antioxidant defence is the CNC-bZIP family. It is composed of four closely related proteins: *p45-NFE2*, *Nrf1*, *Nrf2*, and *Nrf3* [234, 288-290], as well as two distantly related proteins named *Bach1* and *Bach2*. *Bach1* and *Bach2* differ slightly from the other CNC-bZIP proteins in that they have an additional structure referred to as the "broad complex tramtrack bric-a-brac" (BTB) domain [291].

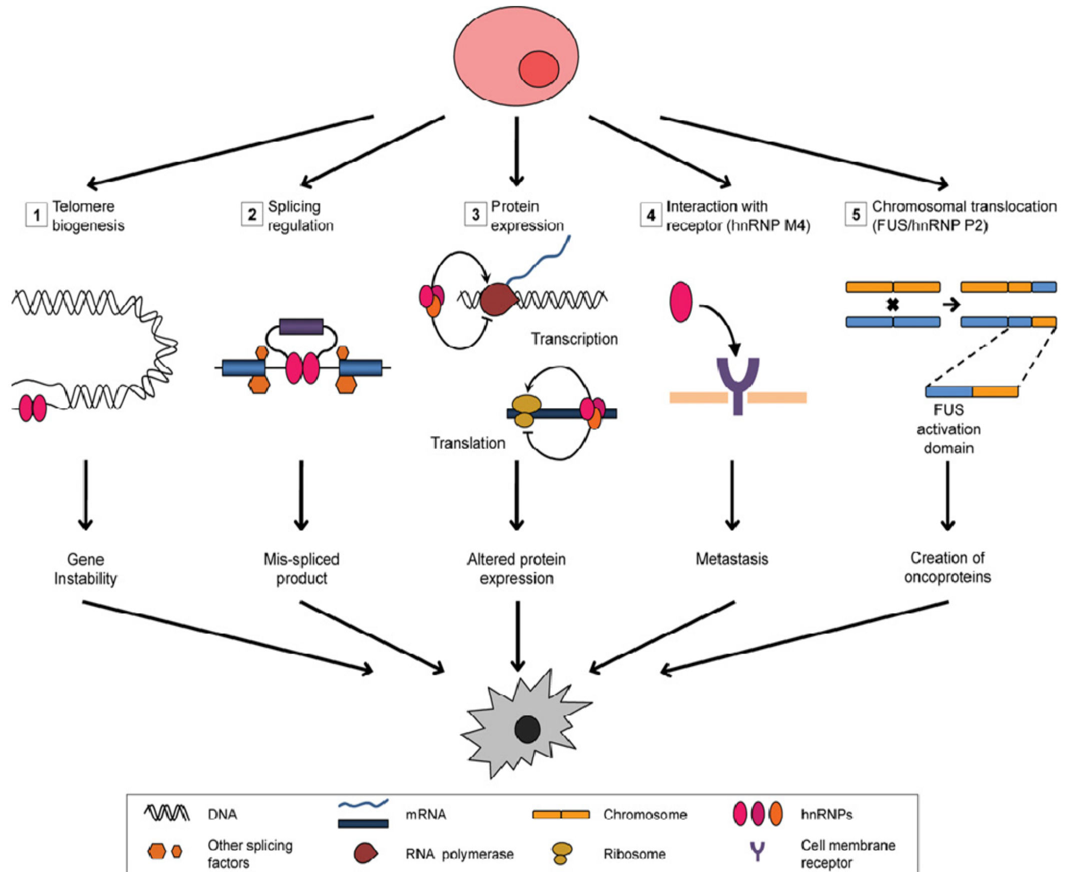

Figure 1-12 HnRNPs can contribute to carcinogenesis, pathways (1)–(3) can involve multiple hnRNPs, whereas pathways (4) and (5) are specific for *hnRNP* M4 and Fus (*hnRNP P2*) respectively. (1) Deviations in telomere biogenesis resultin genome instability. (2) Defects in splicing regulation result in mis-splicing of transcripts involved in cellular proliferation and differentiation, which could alter cell-cycle regulation. (3) Changesin gene expression at the transcriptional or translational level can up- or down-regulate expression of oncogenes or tumour suppressors. (4) *HnRNP M4* interacts with cell membrane receptors totrigger signalling pathways that promote metastasis. (5) Chromosome translocations involving FUS create chimaeric proteins that act as oncoproteins.

1.7.4.1 What is Nrf2?

Nrf2 is a transcription factor that regulates the antioxidant defense in the cells. *Nrf2* transcription factor [292] is a member of the Cap‘n’Collar (CNC) family of bZIP proteins [197, 289]. It was first discovered in the *Drosophila* Cap ‘n’ Collar and was required for labial and mandibular development [293]. *Nrf2* was discovered as a regulator of Globin gene expression in hematopoietic cells [288]. *Nrf2* plays one of the most

important roles in cellular defenses against oxidative stress or electrophiles. *Nrf2* activates the transcription of its target genes by binding specifically to the antioxidant response element (ARE) found in those gene promoters [294]. The antioxidant response element (ARE) is a cis-acting enhancer element located in the 5' flanking region of many phase II detoxifying and antioxidant genes [295]. Many *Nrf2* regulated genes have been identified such as glutamate–cysteine ligase catalytic subunit (GCLC) and heme oxygenase-1 (HO-1)), xenobiotic metabolism enzymes (e.g. NADPH quinone oxidoreductase 1 (NQO1) and Catalase)[296, 297]. The highly conserved C-terminus of *Nrf1*, *Nrf2* and *Nrf3* is similar. It is responsible for DNA binding and heterodimerisation with small *Maf* proteins in the nucleus [298]. On the other hand, the Neh2 and Neh6 domains of *Nrf2* were shown to be a redox-insensitive degron [299]. The gene structure of *Nrf2* is shown in Figure 1-13a.

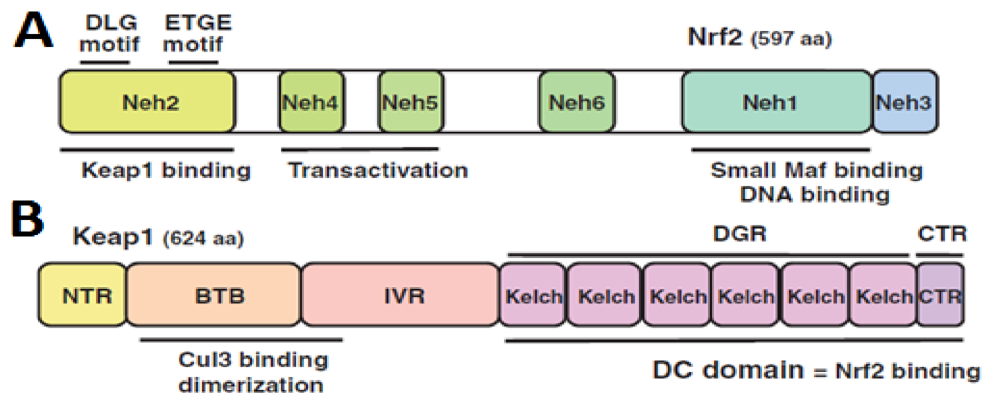

Figure 1-13: Schematic representation for *Nrf2* and *Keap1*domain composition (A) *Nrf2* consists of six highly conserved domains, Neh1 to Neh6 (NRF2-ECH homology: Neh). *Neh2* domain is highly conserved among species and binds with *Keap1*. Neh4 and Neh5 are transactivation domains that bind to CBP. Neh1 is a basic region-leucine zipper structure for dimerization with small *Maf* and DNA binding. (B) Domain structure of *Keap1* protein is as follows; NTR (N-terminal region; a.a. 1-60), BTB (a.a. 61-179), intervening region (IVR; a.a. 180-314), DC domain harboring six Kelch-repeat domain (a.a. 315-359, 361-410, 412-457, 459-504, 506-551, 553-598) and C-terminal region (CTR; a.a. 599-624). BTB domain and N-terminal portion of IVR are regarded important for the association with *Cul3*. DC domain mediates interaction with Neh2 domain of *Nrf2* [300].

1.7.5 *Keap1*

Kelch-like ECH associated protein 1(*Keap1*), discovered in 1999, belongs to the Kelch family, which was identified as an adaptor of *Nrf2* ubiquitination. Murine *Keap1* consists of 624 amino acids with 95% similarity between mouse and human [301] and a high number of cysteine residues, with murine and rat *Keap1* containing 25 cysteine residues and human Keap1 containing 27 cysteine residues.*Keap1* represses the nuclear activation of antioxidant responsive elements by *Nrf2* through binding to the amino-terminal Neh2 domain [302]. *Keap1* consists of different functional domains (Figure 1-13b). The NTR and the BTB domains are essential for homodimerisation of *Keap1*. The intervening region (IVR) is a redox-sensitive cysteine-rich region and the DGRs can interact with actin filaments. Moreover, three conserved arginines (Arg-380, Arg-415, Arg-482) within this domain are reportedly responsible for the binding of *Nrf2* via its Neh2 domain, whereas the BTB and IVR domains are necessary for thermomodulation and degradation of *Nrf2* [302-305] through binding of Cullin 3 (*Cul3*), a ubiquitin ligase adaptor protein [306].

1.7.5.1 *Nrf2* pathways

Under basal conditions, the *Nrf2* protein is negatively regulated in the cytosol by *Keap1*. *Keap1* prevents *Nrf2* translocation to the nucleus by acting as an adaptor to facilitate binding to Cullin 3-based E3 ligase [302]. This complex promotes the degradation of *Nrf2* via the ubiquitin proteasome system. The E3 ubiquitin ligase is one of three enzymes required for protein ubiquitination [307]. The E1 ubiquitin ligase acts as aubiquitin activating enzyme and E2 as a conjugate enzyme, while the E3 substrate adaptor protein complex works cooperatively to attach ubiquitin to a defined protein [308]. The basal level of *Nrf2* maintains the basal gene expression of the cytoprotective enzymes. As *Nrf2* activity is regulated by its degradation and new *Nrf2* is *de novo* synthesized, *Nrf2*

response to the oxidative and electrophilic stress is fast and sensitive. Under oxidative stress and/or electrophilic effect, or under the effect of chemopreventive compounds, *Nrf2* liberate separates from its adaptor protein, *Keap1*, and translocates into the nucleus [309, 310]. The import of *Nrf2* into the nucleus is regulated by its nuclear localization signal (NLS) located in its C-terminus. In the nucleus, *Nrf2* binds to the small *Maf* protein, a co-transcription factor, forming a heterodimer. This heterodimer binds to the ARE region in the promoter of genes involved in phase II detoxification and antioxidant defense [296, 297, 309].

Different mechanisms have been proposed to explain the activation and translocation of *Nrf2*, including *Keap1* modification and *Nrf2* phosphorylation [311, 312]. Under cellular stress, He et al. demonstrate the important role of the *Keap* Cys273 and Cys288 in the suppression of *Nrf2* gene expression [313]. Moreover, the *Bach1* protein competitively and preferentially binds to *Keap1* [314] leading to *Nrf2* release and translocation into the nucleus. Several studies have shown that *Bach1* represses *Nrf2* downstream genes, such as *NQO1* and *GST*, by binding to the ARE and inhibiting ARE-mediated gene expression [314]. It appears that activation of several protein kinases such as PI3K, PKC, JNK, p38MAPK and ERK induce *Nrf2* phosphorylation, facilitating the dissociation of *Nrf2* from *Keap1* and subsequent translocation to the nucleus [315]. It has been shown that PKC induces modification of *Keap 1* at cysteine 151 and phosphorylates *Nrf2* at amino acid serine 40, leading to stabilization and nucleartranslocation of *Nrf2* as well as increased drug resistance [316]. The *Keap1-Nrf2* signaling pathway is illustrated in Figure 1-14.

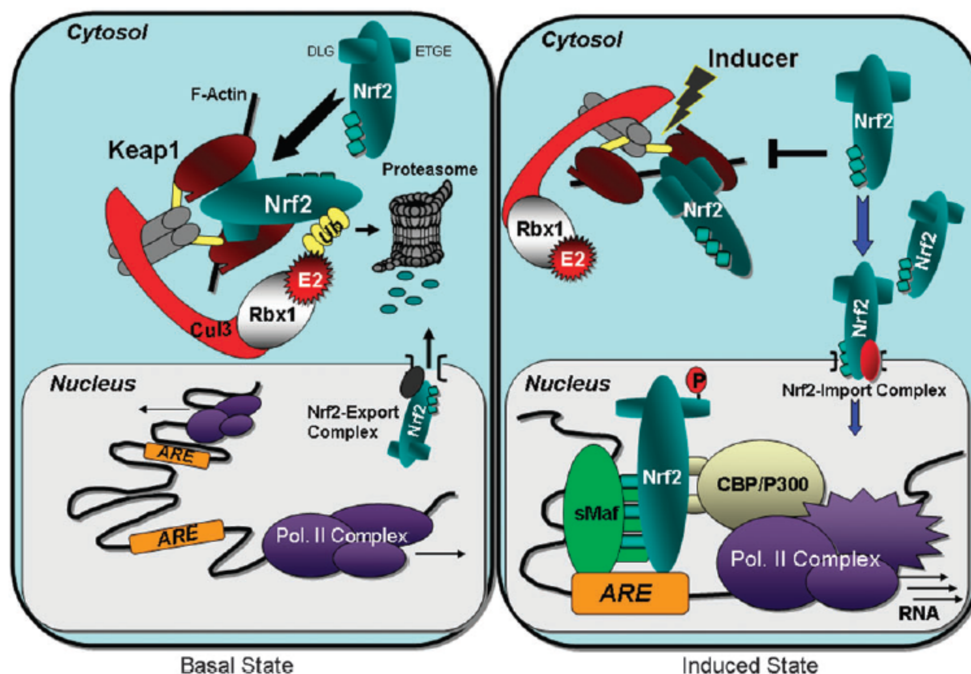

Figure 1-14: General scheme for the induction of cytoprotective genes through the Keap1–Nrf2–ARE-signaling pathway. In the basal state (left panel), *Nrf2* exhibits low steady-state levels and rapid turnover due to ubiquitination and degradation by the proteasome. Chemopreventive inducers (right panel) such as phenolic antioxidants, oltipraz, sulforaphane and triterpenoids increase the nuclear translocation of *Nrf2* primarily through interactions with *Keap1* that impare ubiquitination of *Nrf2* and subsequent proteasomal degradation. Phosphorylation of *Nrf2* by a series of kinases also affects its fate and distribution. After translocation to the nucleus, *Nrf2* transactivates the AREs of cytoprotective genes affecting several protective systems, such as conjugating/detoxication enzymes, antioxidative enzymes, the proteasome, transporters, molecular chaperones and anti-inflammatory pathways [309].

1.7.5.2 Oltipraz and Trigonelline compounds

Oltipraz, [5-(2-pyrazinyl)-4-methyl-1,2-dithiol-3- thione] is a chemopreventive agent for liver and colorectal cancer [317, 318]. Comprehensive mechanistic studies suggest that oltipraz exertscancer chemopreventive effects through the induction of glutathione *S*-transferase (GST) [319].Studies on the mechanisms of action of oltipraz indicate its ability to induce a set of detoxification and antioxidant enzymes through the activation of intracellular signaling mediated by the

nuclear transcription factor named *Nrf2*. Oltipraz treatment disrupts the interaction between *Keap1* (*Nrf2* inhibitor) and *Nrf2*, by liberating *Nrf2* for nuclear translocation and binding to AREs of the antioxidant gene. Moreover, Oltipraz increases the *Nrf2* promoter activity via ARE/EpRE in human hepatoma HepG2 cells [319]. Exposure of experimental animals to oltipraz triggers *Nrf2* expression and translocation to the nucleus [319, 320] as well as enhances *Nrf2*'s antioxidant response element (ARE) binding activity. Cillard's group suggest that oltipraz induced a production of reactive oxygen species that probably acted as second messengers in order to trigger the transcription of many genes [321].Kelley et al. report that weekly oral oltiraz in chronic smokers did not increase mRNA or enzymatic activity of phase II enzymes and resulted in no change in glutathione levels. Thus, despite moderate drug-related toxicity, there was no significant effect on pharmacodynamic or surrogate risk biomarkers [322]. Trigonelline alkaloid is a natural compound present in green coffee beans [323]. Trigonelline alkaloids have been identified to inhibit *Nrf2* translocation [324]. Arlt et al. demonstrated in different pancreatic cell lines that Trigonelline efficiently suppressed *Nrf2* activity upon treatment with tert-butylhydroquinone -induced *Nrf2* activation [325].Trigonelline has hypoglycemic, hypolipidemic, neuroprotective, antimigraine, and anti-tumor activities. Trigonelline alkaloid acts by affecting β cell regeneration, insulin secretion, activities of enzymes related to glucose metabolism, and reactive oxygen species [326].

1.8 Experimental animal models

Experimental animal models in fundamental research are crucial to advancing knowledge. A model provides researchers with practical tools to understand the pathophysiology of disease and to develop drugs for its treatment. Our knowledge of the pathophysiology of diabetic nephropathy has increased after using diabetic rodents. In diabetes, there are several surgical or non-surgical approaches that have been developed to induce diabetes in rodents [327]. The surgical method is the most straightforward way to induce diabetes; the pancreas of the subject of interest is removed in part or completely such that pancreatectomized rats and mice are the most commonly used. The non-surgical methods include either damage to the pancreas (especially the beta cells by chemical administration) or by genetic modification to the insulin gene [328].

1.8.1 Induction of type 1 diabetes

1.8.1.1 Chemical induction

(A) Streptozotocin model of diabetes mellitus

Streptozotocin (STZ) administration has been used to induce hyperglycemia in rodents. STZ is a powerful alkylating agent that interferes with glucose transporters and induces beta cell damage in the pancreas [329]. Although STZ is widely used to induce T1D, it has some disadvantages such as the high incidence of liver and kidney toxicity and the development of tumors [330]. Thus, the data collected from STZ-induced diabetes should be analyzed carefully. Tay’s group has reported that even by careful optimization of the STZ dose while a stable and reproducible diabetic murine modelwas established it was accompanied by renal function impairment and acute tubular necrosis [331]. Therefore, there are significant limitations to STZ-induced diabetes attributable to dosage and duration of the exposure.

(B) Alloxan model of diabetes mellitus

Another popular type 1 diabetes drug inducer is Alloxan, broadly used in mice, rats, rabbits and dogs. Alloxan administration provides a pathological biomodel to investigate the substance with antioxidant activities in vivo [332]. In addition to Alloxan, there are several less commonly used chemicals that induce diabetes, such as Vacor, Dithizone and 8-hydroxyquinolone [333].

1.8.1.2 Genetically manipulated mice

(A)The non-obese diabetic (NOD)

The NOD mouse is a hypoinsulinemia type 1 diabetic model whose renal abnormalities gradually develop with age. In this mouse model, the autoimmune diabetes is caused by destruction of the insulin-producing beta cells in the pancreasat approximately 5 months of age, although the exact age of diabetes onset is somewhat variable[334, 335]. The NOD mouse has proved to be an interesting model of type 1 diabetes and is particularly useful for studying the early features of diabetic nephropathy. Studies of the acute phase of diabetes in the NOD mice showed gradual increases in plasma creatinine with significant changes in the kidney weight as well as mild changes to the glomeruli [336]. It has been reported that in the early stages, NOD mice have high blood pressure, increased GFR, as well as glomerular enlargement, which in the late stage shows severe mesangial expansion and reduced podocyte numbers[337]. Additionally, the RAS is affected; *ACE* activity is higher in the tubules and *ACE2* activity is decreased, leading to a change in the *ACE/ACE2* ratio that may contribute to renal damage [338, 339].

(B)Insulin-2 Akita

Insulin-2 Akita (Ins2Akita) mice are genetically manipulated to develop insulin-dependent diabetes. This model is characterized by more severe hyperglycemia, hypoinsulinemia, polydipsia, and polyuria in males than in

females, which starts around three to four weeks old [340]. The Ins2Akita mutation is autosomal dominant, and is the result of a G-A change at position 1907. This mutation alters the seventh amino acid in the A chain of mature insulin, Cys96 (TGC) to Tyr (TAC). Jackson Laboratories reported that they expected the transition would disrupt a disulfide bond between the A and B chains, inducing a major conformational change in insulin 2 molecules. Homozygous mice for the Ins2Akita allele fail to thrive and die within one to two months [335]. Akita mice are commercially available from Jackson Laboratories. Studies done by Dr. Chan's group and other groups have shown a severe renal histological change in the glomeruli and renal tubules, especially in renal proximal tubules [218, 341]. Chan's group has also shown that at 20 weeks of age the Akita symptoms include hyperglycemia, hypoinsulinemia, albuminuria, and hypertension accompanied by alterations to the RAS system, including gene expression changes in angiotensinogen, RAS enzymes, and receptors [218]. Insulin pellets implanted in Akita mice at week 12 for four weeks almost normalized the glycemic levels, blood pressure, and albumin excretion [341]. The advantage and disadvantage of different type 1 diabetic models are listed in Table 1-7.

(C) Animal models of type 2 diabetes

Type 2 diabetes disorders arise as a result of insulin resistance and impaired insulin secretion. Various animal models have been developed to study type 2 diabetes. *Db/db* and *KKAy* mice are two models of type 2 diabetes widely used to study DN [342, 343]. These two models are characterized by albuminuria, mesangial matrix expansion, and thickening of glomerular capillary basement membrane [344]. Db/db mice were first identified in 1966 [345] . The *db* gene encodes for a point mutation in the leptin receptor, which causes impairment in leptin signaling and leads to hyperphagia, obesity, hyperleptinemia, and hyperinsulinemia [346]. In the rat, Goto Kakizaki (GK) rats are type 2

diabetic models that develop a renal injury similar to human diabetic nephropathy [347]. The GK rat model are characterized as a moderate type 2 diabetes arising at about three to four weeks of age with insulin resistance [348], in addition to a marked renal injury in podocyte and mesangial cells and thickening of the basement membrane [349]. Beside spontaneous or genetically-derived diabetic animals, there are several methods to induce diabetes of diet and/or chemical induction, as well as transgenic/knock-out diabetic animals. Table 1-8 shows different examples of animal models with T2D.

Model category	Strain reported	Advantages	Disadvantages
Streptozotocin	C57BL/6J, C57BLKS, Balb/c, ICR, DBA2, ROP	Well established, reproducible timing; may be established in strains both resistant and susceptible to DN.	Potential for nonspecific toxicity; strain-dependent dosing necessary; biohazard: potential mutagen.
Encephalomyocarditis virus D variant	DBA, Balb-C	May reproduce viral causes of type 1 diabetes in humans; DBA may be prone to DN	Potential for nonspecific renal effects; strain-dependent dosing necessary; biohazard; not widely studied; renal functional effects not characterized
Ins2 Akita	C57BL/6, C3H	Commercially available (JAX); autosomal dominant mutation	Presently only C57BL/6 commercially available; C57BL/6 relatively resistant to nephropathy; hyperglycemia in females is mild
NOD	Inbred line derived from ICR (outbred line)	Spontaneous development of β-cell failure may mimic pathophysiology of disease in humans (99); commercially available	Unpredictable timing of development of diabetes; no appropriate control strain; needs insulin therapy to survive long periods; multigenic-cause diabetes precludes easy intercrosses

Table 1-7: Some mouse models of type 1 diabetes used to study DN [335].

Model category	Type 2 diabetic models	Advantages	Disadvantages
I- Spontaneous or genetically derived diabetic animals	*ob/ob* mouse *db/db* mouse KK mouse Zucker fatty rat Obese rhesus monkey	Diabetes is of spontaneous origin involving genetic factors and the animals develop characteristic features resembling human T2D Mostly of inbred animal in which the genetic background is homogeneous and environmental factors can be controlled, allow genetic dissection of this multifactorial disease easy	Highly inbred, homogenous and mostly monogenic inheritance and development of diabetes is highly genetically determined unlike heterogeneity seen in humans Expensive and Mortality due to ketosis problem is high in case of animals with brittle pancreas (db/db, ob/ob *etc.*) and require insulin treatment in later stage for survival
II- Diet/nutrition induced diabetic animals	Sand rat C57/BL 6J mouse Spiny mouse	Develop diabetes associated with obesity as in diabesit y syndrome of human population Toxicity of chemicals on other body vital organs can be avoided	Require long period of dietary treatment.
III- Chemically induced diabetic animals	GTG treated obese mice	Selective loss of pancreatic beta cells (alloxan/STZ) leaving other pancreatic alpha and delta cells intact Residual insulin secretion makes the animals live long without insulin treatment Ketosis and resulting mortality is relatively less Comparatively cheaper, easier to develop and maintain.	Hyperglycaemia develops primarily by direct cytotoxic action on the beta cells and insulin deficiency rather than consequence of insulin resistance. Chemical produce toxic actions on other body organs as well besides its cytotoxic action on beta cells Variability of results on development of hyperglycaemia is perhaps high
IV- Surgical diabetic animals	VMH lesioned dietary obese diabetic rat	Resembles human type 2 diabetes due to reduced islet beta cell mass	Problems as a result of part of excision of exocrine portion (deficiency of amylase enzyme) Dissection of alpha islets (glucagon secreting cells) too along with beta cells leading to problems in counter regulatory response to hypoglycaemia Mortality is comparatively higher
V- Transgenic/knock -out diabetic animals	β3 receptor knockout mouse	Effect of single gene or mutation on diabetes can be investigated *in vivo* Dissection of complex genetics of type 2 diabetes become easier	Highly sophisticated and costly procedure for the production and maintenance. Expensive for regular screening experiments

Table 1-8; Advantages and disadvantages of different categories of type 2 diabetic animal models [350].

Genetic and environmental interactions contribute to the development of hypertension [351]. Animal models are vital for studying the genes involved in the pathogenesis of hypertension disease. The perfect animal model should resemble hypertension in humans. There is no single species that can answer all the questions that are raised in understanding the etiology and regulation of hypertension [352]. Thus, several animal models are required to answer some individual hypertension traits. Understanding the etiology of the hypertension in the animal model will lead to developing treatments to control increased hypertension.

1.8.2 Mouse models of hypertension

Hypertension can be induced in animals in several ways, including by surgical induction in dogs. Deoxycorticosterone acetate-salt (DOCA-salt) is a common endocrine method to induce hypertension. The generation of hypertension usually requires partially removing some renal mass in addition to DOCA administration and a high-salt diet [353]. Dahl salt-sensitive rats develop salt-sensitive hypertension and organ damage [354, 355]. Zhuo et al. have shown that ANG II infusion induce hypertension mediated through AT1 receptor [356].

1.8.2.1 Genetically modified animal models

Genetic engineering techniques are used as tools to develop new types of animal models called transgenic or knockout models. Transgenic refers to the overexpression of a foreign gene, usually from a different species, that targets certain organelles, cells, and/or tissues [352]. Contrary to a transgenic model, a knockout model is characterized by the inactive, or "knocked out," endogenous gene by its replacement or disruption [357].

1.8.2.2 Transgenic models of the Renin Angiotensin System

The Murine Renin (*Ren2d*) gene was introduced into the rat's genome. *Ren2d*-Tg rats developed high blood pressure by eight weeks of age [358]. In that model, administering RAS blockers halted the increase in systolic blood pressure [359]. Bohm et al. suggested that the augmentation of blood pressure was due to an increase in the formation of local ANG II [359]. The same transgene was introduced into a single renin-gene mouse (*Ren1c*). Surprisingly, it did increase the blood pressure of the mice. The differences in the kinetics of angiotensin cleavage by renin in the two species account for the differences in observations [360, 361].

The renin substrate has been described as a gene that potentially underlies the development of hypertension [362] and the human angiotensinogen gene has been cloned into animal models studying this. In situ hybridization of the hAgt-Tg mice shows that human *Agt* mRNA is expressed in the renal proximal tubules, especially in the epithelial cells [363]. Similarly, transgenic mice with rat angiotensinogen exhibit an increase in systolic blood pressure. Studies by Chan's group in which angiotensinogen was overexpressed in the renal proximal tubules driven by the mouse's KAP2 promoter reported that overexpressing rat *Agt* in the mouse renal proximal tubule cells resulted in kidney injury, increased albumin excretion in the urine, and high blood pressure. Moreover, RAS blockade decreases blood pressure and proteinuria in transgenic mice overexpressing rat angiotensinogen gene in the kidney [166]**.** Human*AT1R* has been introduced into mice under the control of an alpha-myosin heavy chain promoter. The mice overexpressed *AT1R* in cardiomyocytes and showed cardiac hypertrophy with interstitial collagen deposition and died prematurely of heart failure [364]. Meanwhile, overexpressing human *AT1R* in rat podocytes, under the control of a

nephrin promoter, resulted in increased albuminuria around 8-15 weeks of age [365]. Moreover, damage in the glomerulus was reported as a result of the formation of pseudocysts in the podocytes that were followed by foot process effacement and local detachments enhancing nephron loss [365]. Taken together, we conclude that overexpression of *AT1R* in cardiomyocytes or podocytes causes tissue damage and could consequently lead to death [366].

AT2R transgenic mice were generated using the alpha-myosin heavy chain promoter [367]. Compared to the wild type, *AT2R* expression was higher in the hearts of the transgenic mice while there was no significant change in blood pressure. When ANGII infusion was introduced into the wild type and AT2-Tg, the transgenic mice's blood pressure decreased compared to the wild type. It has been shown that overexpression of *AT2R* under the control of the alpha-myosin heavy chain promoter leads to a decrease in AT1R sensitivity [368].

Human *ACE* cDNA has been used to generate h*ACE*-Tg rats. *ACE* was expressed in the cardiac ventricles under the control of a 2.1-kb rat myosin light chain-2 (*rMLC-2*) promoter [369]. Under normal conditions, no significant change was noted between the wild type and the transgenic model. However, induction of hypertension resulted in more damage in the transgenic mice compared to the wild type. In addition, there was increased cardiac hypertrophy as well as induction of ANF and collagen III expression compared to the wild type [369]. As mentioned in section 1.5.3, *ACE2* acts on its substrate ANG II to produce Ang-(1-7), exerting effects that are opposite to those of ANG II mediated by the *MAS* receptor [370]. Spontaneously hypertensive stroke-prone rats (SHRSP) are hypertensive with low *ACE2* gene expression [371]. Michael Bader's group has generated *ACE2* transgenics expressing human *ACE2* in rat

vascular smooth muscle under the control of the SM22 promoter [371]. The transgenic rats were produced in a SHRSP genetic background. The phenotype of these mice indicated low blood pressure compared to the SHRSP control group. Moreover, the administration of ANG II to SHRSP-*ACE2*-Tg and SHRSP rats attenuated arterial blood pressure in transgenic rats compared to the non-transgenic rats with the same background [371]. Based on these studies, the promise of transgenic mice for the study of *ACE2* and as potential targets for therapeutic interventions in hypertension is sustained [372].

1.8.3 Knockout models and RAS system

Knock out or “gene suppression” is another strategy used to study causative genes that are essential for hypertension development. Suppression of gene expression is used to examine the physiological effects of disrupting basal gene expression, which could reveal the fundamental function of the gene. There are different types of gene suppression referred to as knock-out, knock-down, and knock-in [373]. Through these techniques, a number of models have been produced to understand the multifactorial causes of essential hypertension. Knocking out the *Ren2* gene in mouse strains (129/Ola) had no significant effect on blood pressure, while the *Ren1* knockout causes reduction of blood pressure in the females of both strains [374, 375]. Knocking out angiotensinogen results in hypotensive mice [376]. Similarly, the *ACE* knockout gene results in hypotensive mice, especially in males [377]. Interestingly, studies on the SNGFR of *ACE* knockout mice indicate that TGF is absent in *ACE*-deficient mice but can be restored by acute infusion of ANG II [378]. Deleted *ACE2* from the X chromosome in mice results in an impairment in heart function [379]. The loss of *ACE2* results in cardiac contractility deficiency, increased angiogenesis II levels, and upregulation of hypoxia-induced genes in the heart [380]. Angiotensin II receptors have been knocked out in the mouse; the *AT1R-$_A$* knockout

resulted in hypotensive mice accompanied with no TGF response [381] and showed hypertrophy of the JGA, while mice deficient in $AT1_B$ had normal phenotypes. These results suggest that *AT1a* can compensate for the role of $AT1_B$receptors [381].

1.8.4 Therapy

Several studies demonstrate local RAS activation in different organs such as lipids [382], heart [145, 383], kidneys [128] and the vascular system [384] in diabetics. Angiotensinogen is mainly produced in the liver, while the kidney is the major source of renin [385]. ACE produced in the lung [386] (RAS) participates significantly in the pathophysiology of hypertension, congestive heart failure, myocardial infarction, and diabetic nephropathy [387]. In diabetic nephropathy, the use of antihypertensive drugs slows the progression of the disease with the control of hyperglycemia [388, 389]. The RAS blockade focuses mainly on disturbing the RAS signal either by targeting its enzymes, such as renin and/or ACE, or by blocking the ANG II receptor (AT1) [389]. Inhibitors of the RAS are capable of lowering BP. Inhibition of renin activity results in a decreased angiotensin I production, consequently suppressing the generation of active peptide angiotensin II. For example, Aliskiren is a drug used to directly inhibit renin activity [390]. Renin inhibitors show antihypertensive and antialbuminuric effects in humans and in animal models [391]. The antihypertensive effects of the direct renin inhibitor Aliskiren lasts substantially longer after treatment withdrawal than expected based upon its plasma half-life [392]. It is thought that since the main producer of circulatory renin is the kidney, and Aliskiren accumulates in the kidney, it lasts for a longer time following treatment cessation than expected based upon its plasma half-life [393].

Hypertension, diabetes and hypercholesteremia conditions are associated with activation of ACE tissue that disrupts vasodilation/vasoconstriction balance. Pathological activation of tissue ACE results in increases of local Ang II [394]. ACE inhibitors (ACEi) have been shown to effectively lower blood pressure and play a significant role in renal and cardioprotective effects. ACEi decrease the biosynthesis of Ang II but do not inhibit the non-ACE ANGII generating pathway [395] (Figure 1-14). ACE inhibitors are classified based on their lipophilicity index and rate of dissociation from ACE in vitro. ACEi inhibitors such as Ramipril and Trandolapril have high tissue affinity with a high lipophilicity index and slow dissociation from ACE while Enalapril and Captopril have low tissue affinity [396, 397]. It has been proposed that high affinity tissue may result in better vascular protection. In renal disease, ACEi treatment promotes reduction of blood pressure and proteinuria, which are important biomarkers of renal disease [398]. In addition to the Angiotensin I substrate, ACE has different substrates, which include Bradykinin, substance P, and other Tachykinins [399]. Thus, some clinicians have reservations about using ACEi because of accumulation of ACEi substrates.

The biologically active Ang II protein interacts with at least two known membrane receptors. A continual increase of Ang II levels results in binding to *AT1R*, consequently increasing blood pressure, renal damage, and myocardial hypertrophy. Ang II production is formed not only by the action of the ACE on Angiotensin I but also via pathways involving cathepsin G, elastase, tissue plasminogen activators, chymostatinsensitive AT-II-generating enzymes, and chymase [400]. Therefore, ACEi are only partial inhibitors that reduce the formation of Ang II, setting the stage for the development of a specific and selective blocker. The angiotensin receptor blockers (ARBs) were developed to completely block the RAS and decrease the adverse effects seen with

ACE inhibitors. In contrast to ACE inhibitors, ARBs allow activation of AT2 receptors [401, 402]. Several ARBs currently on the market that are used for hypertension and cardiovascular complications include Losartan, Valsartan, Candesartan, Eprosartan, and Irbesartan [402]. Losartan is a selective oral *AT1R* antagonist. It is an approved drug for diabetic nephropathy treatment. Garcha et al. showed that Losartan is more selective towards binding AT1 than AT2 receptors by 30,000 fold [403]. ARBs result in a decrease in endothelial dysfunction, a reduction of proteinuria, and the preservation of kidney function in diabetic patients [404]. In some patients whose blood pressure was poorly controlled by either ACEi or ARBs, a combination treatment with the two inhibitors is recommended in order to increase the antihypertensive efficacy in those patients. It has been reported that ACEi or ARBs are ideal antihypertensive agents in patients with CKD [405]. The *ACE / ACE2* balance affects hypertension development, Figure 1-15.

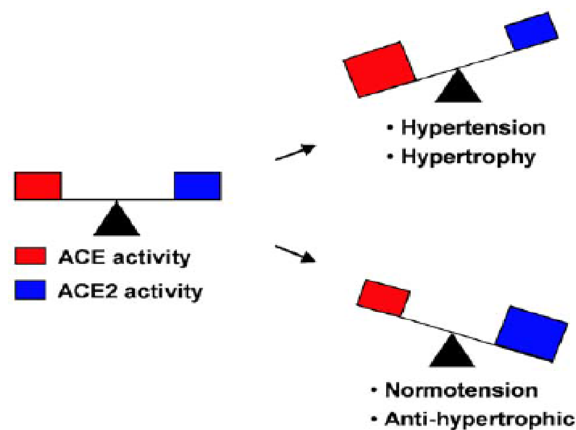

Figure 1-15; schematic diagram shows the balance between ACE and ACE2.

ACE2 is expressed in different organs including the lungs, stomach, spleen, intestine, bone-marrow, kidney, liver, and the brain as a membrane-bound protein [406]. *ACE2* degrades ANG II to angiotensin (1-7)[406]. Interestingly, ACEi can increase Ang-(1–7) levels, mimicking the

action of *ACE2* on Ang II [395]. Thus, *ACE2* has been suggested as a candidate for the development of new therapeutic strategies [406]. Recently, human recombinant *ACE2* (hr*ACE2*) has been developed and used to study its efficiency at degrading ANG II in vivo and in vitro. Studies showed that *ACE2* can rapidly metabolize Ang II by lowering the plasma Ang II levels and increasing angiotensin-(1-7) levels [407, 408]. In C57BL/6J mice, the increase in blood pressure triggered by Ang II infusion was completely blocked by the administration of recombinant ACE2 [407].

The disadvantages of antihypertensive drugs include undesired side effects, the lack of specificity, and a short lifetime [409]. Moreover, these types of drugs deal with the symptoms of the disease rather than addressing the causes of disease. Gene therapy is a novel approach that results from the integration of advances in technology and molecular biology techniques. Gene therapy approaches provide us with a possibility of producing a long term therapy, which is tailored to the patient's own genetic material. Several studies have been exploring these technologies by transferring the coding regions of vasodilatory genes into hypertensive animal models such as Kallikrein [410, 411], and showed reductions in blood pressure. The second approach includes silencing the genes involved in vasoconstrictor responses under constitutive promoters [412]. The gene therapy field holds many promises but requires more investigation, particularly with regard to how to deliver a gene to its intended target.

1.9 Objectives and hypothesis of this study

In recent years, Chan's group has used invitro and invivo models to establish the links between stressinduced in the proximal tubules and the progress of kidney diseases such as diabetes and hypertension. The central aim of this thesis is to identify the molecular mechanism(s) underlying the inhibition or induction of renal *Agt* by insulin treatment or catalase overexpression, respectively, in RPTs of type 1 diabetic mice. In addition, the aim is also to elucidate the possible pathway(s) that might be involved in *Agt* regulation in ROS activation. Invitro studies have shown that insulin inhibits the stimulatory effect of high glucose levels on r*Agt* gene expression and the induction of hypertrophy in IRPTCs [153, 413-415]. Additionally, Wie et al [229, 230] have identified an insulin-responsive element (IRE) in the rat *Agt* gene promoter that binds to *hnRNP F* and *hnRNP K*. In chapter 2, we hypothesized that insulin prevention of hypertension and kidney injury is mediated viainhibition of renal *Agt* gene expression and upregulation of *hnRNP F* and *hnRNP K* expression in the Akitamouse model of type 1 diabetes.

Aim I

The aim was to investigate the contribution of *hnRNP F* and *hnRNP K* on insulin inhibition of renal *Agt* gene expression, prevention of hypertension, and kidney injury in Akita mice.In addition, the aim was also to elucidate the possible mechanism of action underlying insulin activation of *hnRNP F* / *hnRNP K* and *Agt* downregulation.

ROS mediate high-glucose stimulation of angiotensinogen gene expression in RPTCs invitro [152, 416]. Catalase (*Cat*) overexpression attenuates hypertension and RPTC apoptosis in non-diabetic *Agt/Cat*-Tg [219] and diabetic *Cat*-Tg mice [417]. Protection against oxidative damage is accomplished by a complex defense system composed of antioxidant molecules (such as *Nrf2*) and antioxidant enzymes (SOD, GSH and Catalase) that convert excessive reactive species to less reactive and less damaging forms [418]. Several reports indicate that the persistent accumulation of *Nrf2* in the nucleus is harmful. Under oxidative or electrophilic stress, Maher et al. [419] reported that *Nrf2* regulates the expression of several multidrug resistance-associated proteins, which could lead to chemotherapeutic drug resistance. Little information is available about the functional relationship between ROS, *Nrf2* and *Agt* gene expression in diabetic RPTCs, which may be critical for development of diabetic renal injury. In chapter 3 we hypothesize that hyperglycemia, with hypo-insulinemia and low catalase activity, induces an ROS activation that exerts a stimulatory effect on renal *Agt* through *Nrf2* activation; whereas *Cat* overexpression in RPTs of Akita mice attenuates *Nrf2-Agt* activation axe, systolic blood pressure, and kidney injury of diabetic mice.

Aim II

This study investigated the impact of catalase overexpression in RPTCs on *Nrf2* stimulation of *Agt* gene expression and development of hypertension and renal injury in diabetic Akita transgenic mice.

Chapter 2: Article 1

Heterogeneous Nuclear Ribonucleoprotein F and K Mediate Insulin Inhibition of Renal Angiotensinogen Gene Expression andPrevention of Hypertension and Kidney Injury in Diabetic Mice

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Heterogeneous Nuclear Ribonucleoprotein F and K Mediate Insulin Inhibition of Renal Angiotensinogen Gene Expression and Prevention of Hypertension and Kidney Injury in Diabetic Mice

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ABSTRACT

Aims/hypothesis We investigated whether heterogeneous nuclear ribonucleoprotein F and K (hnRNP F/K) mediate insulin inhibition of renal angiotensinogen (*Agt*) gene expression and prevention of hypertension and kidney injury in type I diabetic Akita mice.

Methods Adult male Akita mice (12 weeks of age) were treated +/- insulin implants and euthanized at week 16. Untreated non-Akita littermates served as controls. The insulin effects on blood glucose, systolic blood pressure (SBP), renal proximal tubular cell (RPTC) gene expression and interstitial fibrosis were studied. We also examined immortalized rat RPTCs stably transfected with control plasmid or plasmid containing rat *Agt* gene promoter *in vitro.*

Results Insulin treatment normalized blood glucose levels and SBP and inhibited renal Agt expression but enhanced hnRNP F/K and angiotensin-converting enzyme 2 (Ace2) expression, attenuated renal hypertrophy and glomerular hyperfiltration, decreased urinary albumin/creatinine ratio, as well as Agt and Ang II levels in Akita mice. *In vitro*, insulin inhibited *Agt* but stimulated *hnRNP F/K* gene expression in high glucose media via p44/42 mitogen-activated protein kinase signaling in RPTCs. Transfection with *hnRNP F* or *hnRNP K* small interfering RNA prevented insulin inhibition of *Agt* gene expression in RPTCs.

Conclusions/interpretation These data indicate that insulin prevents hypertension and attenuates kidney injury, at least in part, through suppressing renal *Agt* gene transcription via up-regulation of hnRNP F/K expression in diabetic Akita mice. HnRNP F/K may be potential targets in the treatment of hypertension and kidney injury in diabetes.

Keywords:
Akita Mice, Angiotensinogen, Angiotensin-converting enzyme 2, Diabetes Heterogeneous nuclear ribonucleoprotein F, Heterogeneous nuclear ribonucleoprotein K, Hypertension Insulin, Kidney Injury

ABBREVIATIONS

ACR, albumin-creatinine ratio; ACE, angiotensin-converting enzyme; Ace2, angiotensin-converting enzyme-2; Agt, angiotensinogen; Ang II, angiotensin II; Ang 1-7, angiotensin 1-7; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; GFR, glomerular filtration rate; hnRNP F, heterogeneous nuclear ribonucleoprotein F; hnRNP K, heterogeneous nuclear ribonucleoprotein K; rAgt, rat angiotensinogen; RAS, renin-angiotensin system; RPTs, renal proximal tubules; RPTCs, renal proximal tubular cells; RT-qPCR, real time-quantitative polymerase chain reaction; SBP, systolic blood pressure; siRNA, small interfering RNA; Tg, transgenic; TGF-β1, transforming growth factor-beta 1; WT, wild type

INTRODUCTION

Intensive insulin therapy is the most effective treatment for preventing the progression of nephropathy in type 1 diabetes, though the underlying mechanisms remain incompletely understood [1-3]. The existence of a local renin-angiotensin system (RAS) within the kidney is well established [4]. Renal proximal tubular cells (RPTCs) express all components of the RAS [5-7]. Intrarenal RAS gene expression and urinary angiotensinogen (Agt, the sole precursor of angiotensins) levels are elevated in diabetic and hypertensive animal models and in humans with hypertension and/or diabetes [8-12]. Transgenic (Tg) mice specifically overexpressing murine Agt in their RPTCs develop hypertension, albuminuria, and tubular apoptosis [13-15]. Moreover, renal Agt overexpression and hyperglycemia act in concert to enhance hypertension and kidney injury in diabetic mice [16,17] , indicating important roles for intrarenal RAS activation and hyperglycemia in the development of hypertension and kidney injury in diabetes.

We previously reported that insulin inhibits high-glucose stimulation of renal rat *Agt* (*rAgt*) gene expression and RPTC hypertrophy via a putative insulin-responsive element (IRE) in the *rAgt* gene promoter [18-20] that interacts with 2 nuclear proteins, heterogeneous nuclear ribonucleoprotein F and K (hnRNP F/K) *in vitro* [21, 22]. Recently, we observed that hnRNP F overexpression inhibited renal Agt expression and attenuated hypertension and kidney hypertrophy in Akita (type I diabetic mouse model) Tg mice specifically overexpressing hnRNP F in their RPTCs [23]. These findings suggest that

hnRNP F either acts alone or interacts with hnRNP K or other transcriptional factors to inhibit renal *Agt* gene transcription in diabetes.

The aim of the present study was to investigate the contribution of hnRNP F/K to insulin inhibition of renal *Agt* gene expression and prevention of hypertension and kidney injury in Akita mice.

MATERIALS AND METHODS

Reagents

D-glucose, D-mannitol, human insulin, PD98059 (an inhibitor of p44/42 mitogen-activated protein kinase (p44/42 MAPK)), wortmannin and Ly 294,002 (specific inhibitors of phosphatidylinositol 3-kinase (PI3-K)) and anti-β-actin monoclonal antibody were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). U0126, an inhibitor of p44/42 MAPK, was from Cell Signaling Technology (New England Biolabs Ltd, Whitby, ON, CA). Normal glucose (5 mmol/l D-glucose) Dulbecco's Modified Eagle's Medium (DMEM, catalogue no. 12320), penicillin/streptomycin and fetal bovine serum (FBS) were procured from Invitrogen, Inc. (Burlington, ON, Canada). Insulin implants (Linβit contains insulin of bovine, porcine or human origin (NB: species of origin is not specified by the manufacturer) with a release rate of approximately 0.1 unit/implant/d for >30 d) were bought from Linshin (Scarborough, ON, Canada). The pGL4 [Luc/Puro] vector containing the luciferase reporter was obtained from Promega Corp. (Sunnyvale, CA, USA). *rAgt* gene promoter (N-1498 to N+18) [24] was cloned from rat genomic DNA by conventional polymerase

chain reaction with specific primers (gene number NW_047536.21; sense primer: AAA GGT ACC AGT CTC TCT GGT CAC TAC CCA T and anti-sense primer: AAA AAG CTT GCC CAG ACA AGC ACA GCT AT) and inserted into pGL4 vector via Kpn I and Hind III restriction sites. Rat cAMP-responsive element binding protein (CREB) cDNA was cloned in our laboratory (J.S.D.C.) and inserted into pRSV vector (25). Rabbit polyclonal antibodies specific to hnRNP F (CTARRYIGIVKQAGLER corresponding to amino acids 215 to 230 of mouse and human hnRNP F) [21], and polyclonal antibodies against rAgt were generated in our laboratory (J.S.D.C.) [26]. The following antibodies were used: rabbit polyclonal anti-hnRNP K, anti-transforming growth factor-beta 1 (TGF-β1), anti-ACE antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA), anti-Ace2 antibody (R&D Systems, Inc., Minneapolis, MN, USA) and monoclonal anti-collagen type IV antibody (Chemicon International, Inc.,Temecula, CA, USA). Scrambled Silencer Negative Control # 1 siRNA and *hnRNP F/K* siRNA were obtained from Ambion, Inc. (Austin, TX, USA) [22]. Oligonucleotides were synthesized by Invitrogen, Inc. Restriction and modifying enzymes were obtained from Invitrogen, Inc. and Roche Biochemicals (Laval, QC, Canada).

Physiological Studies

Adult male heterozygous Akita mice with a mutated *insulin2* gene (C57BL6-Ins2^{Akita}/J) were purchased from Jackson Laboratories (Bar Harbor, ME, USA: http://jaxmice-jax.org). Akita mice (10 weeks of age) were divided

into 2 groups with or without insulin implants at week 12 (8 mice per group), as described previously [16]. Briefly, diabetic mice received a subcutaneous insulin implant (2 Linβit implants per 20 g body weight (BW) and a 1 Linβit implant for each additional 5 g BW). Blood glucose levels were determined with small drops (about 2-3 μl/drop) of blood collected from the tail vein following 4-5 hrs fasting with an Accu-Chek Performa System (Roche Diagnostics, Laval, QC, Canada). Unless otherwise noted, data were collected from male Akita mice aged 10-16 weeks. Non-Akita littermates of the same age and sex served as wild type (WT) controls. All animals were fed standard mouse chow and water *ad libitum*. Animal care and procedures were approved by the CRCHUM Animal Care Committee and followed the Principles of Laboratory Animal Care (NIH publication no. 85-23, revised 1985: http://grants1.nih.gov/grants/olaw/references/phspol.htm).

Systolic blood pressure (SBP) was measured with a BP-2000 tail-cuff pressure monitor (Visitech Systems, Apex, NC, USA) [13, 14, 16, 17, 23] in the morning, at least 2-3 times a week, for 5 weeks. The mice were accustomed to the procedure for at least 15-20 min per day for 5 days before the first SBP measurements. SBP values represent the mean ± SEM of 2-3 determinations per week per animal per group. Twenty-four h prior to euthanasia, BW was recorded and mice were housed individually in metabolic cages. Blood from individual mouse was collected (~ 500 to 1,000 μl) by intra-cardiac exsanguination prior to euthanasia and then centrifuged to obtain serum. Urine (~100 to 400 μl/mouse) was collected and assayed for albumin (enzyme-linked immunosorbent assay

(ELISA), Albuwell, Exocell, Inc., Philadelphia, PA, USA) and creatinine (Creatinine Companion, Exocell, Inc.) [13, 14, 16, 17, 23].

The glomerular filtration rate (GFR) was estimated according to the protocol described by Qi et al. [27], as recommended by the Animal Models of Diabetic Complications (AMDCC) (http://www.diacomp.org/) with slight modifications [28]. Briefly, each mouse received a single intravenous bolus of 5% fluorescein isothiocyanate-inulin (FITC-inulin), followed by collections of blood samples (each ~20 μl) from the saphenous vein at 3, 7, 10, 15, 35, 55, and 75 min post-FITC-inulin injection. Plasma fluorescence concentration was measured by Synergy 2 fluorometer (BioTek Instruments, Inc., Winooski, VT, USA) with 485 nm excitation and read at 538 nm emission. GFR was calculated according to the following equation: $GFR = I/(A/\alpha + B/\beta)$, where I is the amount of FITC-inulin bolus delivered, A and α are the y intercept and decay constant of the rapid (initial) decay phase, respectively, and B and β are the y intercept and decay constant of the slow decay phase, respectively [27].

The kidneys were removed immediately after GFR measurements, decapsulated and weighed before Percoll gradient isolation of renal proximal tubules (RPTs) [13, 14, 16, 17, 23]. Aliquots of freshly-isolated RPTs from individual mice were immediately processed for total RNA or protein isolation. The remaining RPTs were then pooled, suspended in normal glucose (5 mmol/l D-glucose plus 20 mmol/l D-mannitol) or high glucose (25 mmol/l D-glucose) DMEM containing 1% depleted FBS ± insulin (100 mmol/l) [18-22], and incubated *ex vivo* at 37ºC for 16 h. At the end of the incubation period, RPTs

were harvested and assayed for *Agt*, *hnRNP F*, *hnRNP K* and *Ace2* mRNA expression.

Serum and Urinary Agt and Ang II Measurement

Serum and urinary Agt were quantified by ELISA (Immuno-Biological Laboratories Inc. Minneapolis, MN, USA) [23]. To measure Ang II levels, serum and urine samples were extracted using a kit and were assayed by specific ELISA for Ang II (Bachem Americas Inc., Torrance, CA, USA) [14, 17, 23].

Morphologic Studies

Kidneys were collected in Tissue-Tek cassettes (VWR Canlab, Montreal, QC, Canada), dipped immediately in ice-cold 4% paraformaldehyde, fixed for 24 h at 4°C, and then processed by the CHUM Pathology Department. Tissue sections (3-4 μm thick, 4-5 sections per organ) were counterstained with periodic acid Schiff (PAS) or Masson's trichrome [14, 16, 17, 23] and analyzed under light microscopy by 2 independent investigators blinded to the treatments.

The tubular luminal area, mean glomerular and RPTC volumes were assessed as described previously [14, 16, 17, 23].

Immunohistochemical staining of Agt, hnRNP F, hnRNP K, Ace2, ACE, TGF-β1 and collagen IV was performed using a standard avidin-biotin-peroxidase complex method on 4 to 5 sections per kidney and 3 mouse kidneys per group (ABC Staining; Santa Cruz Biotechnology) [13, 14, 16, 17, 23]. Staining was analyzed under light microscopy by 2 independent investigators

blinded to treatment groups. TGF-β1- and collagen IV-immunostained images were quantified with National Institutes of Health ImageJ software [13, 14, 16, 17, 23]. Immunostaining with non-immune normal rabbit serum in non-Akita mouse kidneys served as controls, and showed no staining (pictures not shown).

Effect of Insulin on Agt, hnRNP F and hnRNP K Expression in Rat RPTCs

Immortalized rat RPTCs from passages 12 to 18 [6] that express the mRNA and protein of RAS components mimicking RPTCs *in vivo* were used. The plasmids pGL4-*Agt N-1498/+18* were transfected into rat RPTCs, and stable transformants were selected in the presence of 0.6 mg/l of puromycin (Sigma-Aldrich Canada Ltd.) according to the limiting dilution method [29].

To study the effect of insulin, 75-85% confluent stable transformants were synchronized overnight in serum-free 5 mmol/l D-glucose DMEM, then incubated in normal glucose (5 mmol/l D-glucose plus 20 mmol/l D-mannitol) or high-glucose (25 mmol/l D-glucose) DMEM containing 1% depleted FBS and various concentrations of insulin (10^{-13} to 10^{-5} mol/l or 0.1 pmol/l to 10 μmol/l) for 24 h ± PD 98059 (1×10^{-5} mol/l) or Ly 294,002 ((1×10^{-6} mol/l) or wortmannin (1×10^{-6} mol/l) or U0126 (1×10^{-6} mol/l) as described previously [18-20]. Promoter activity was measured by the luciferase activity assay [28]. RPTCs stably transfected with the plasmid pGL4 served as controls.

In additional studies, stable transformants (RPTCs) were transfected with scrambled siRNA or *hnRNP F* siRNA or *hnRNP K* siRNA [22] or with pRSV (empty vector), pRSV-*CREB*, pRSV-*hnRNP F* and /or pRSV-*hnRNP K* and the

effects of insulin on *Agt* gene promoter activity and *Agt* mRNA expression were analyzed after 24 h of incubation.

Real Time-Quantitative Polymerase Chain Reaction (RT-qPCR) Assays and Western Blotting (WB)

Agt, hnRNP F, hnRNP K, ACE, Ace2 and β-*actin* mRNA expression in mouse liver, isolated RPTs and cultured rat RPTCs was quantified by RT-qPCR using forward and reverse primers corresponding to their cDNA sequence as described previously [17, 21-23].

WB was performed as described previously [21-23]. The relative densities of Agt, hnRNP F, hnRNP K and β-actin bands were quantified by computerized laser densitometry (ImageQuant software (version 5.1), Molecular Dynamics, Sunnyvale, CA, USA).

Statistical Analysis

Statistical significance between the experimental groups was analyzed by 1-way ANOVA (analysis of variance) and subsequent Bonferroni test. Values are expressed as mean ± SEM. $p<0.05$ values were considered to be statistically significant.

RESULTS

Physiological Parameters in Mice

Blood glucose levels were significantly elevated in Akita mice compared to wild type (WT) controls (**Figures 1a** and **Table I**). Insulin treatment decreased though never completely normalized blood glucose levels in Akita mice. Average SBP was significantly higher (by 20 to 25 mm Hg) in Akita mice at age 11 weeks than in WT mice ($p<0.005$) (**Figure 1b** and **Table I**) and remained higher for the duration of the study. Insulin treatment completely normalized SBP in Akita mice.

Kidney size, kidney weight (KW, both kidneys), KW/body weight (BW) ratio and heart weight (HW)/BW ratio were higher in Akita than WT control mice. Insulin treatment normalized KW/BW ratio and attenuated HW/BW ratio in Akita mice (**Table I**).

Albumin/creatinine ratio (ACR; **Figure 1c**) and GFR (**Figure 1d**) were significantly higher in 16-week-old male Akita than in WT mice, and these changes were normalized by insulin treatment.

Urinary levels of Ang II (**Figure 1e**) and Agt **(Figure 1f)** were significantly higher in Akita than WT mice. Insulin treatment normalized urinary Ang II levels and partially attenuated urinary Agt levels in Akita mice.

Histologic and Functional Studies

Unlike WT controls, Akita mice developed renal structural damage (**Figure 2a**).

Histologic findings included tubular luminal dilation with an accumulation of cell debris in the tubular lumen. Insulin treatment of Akita mice markedly reversed, though never completely resolved these abnormalities.

Moreover, renal tubular luminal area, glomerular tuft volume and RPTC volume were significantly increased in Akita mice compared to WT mice (**Table I**). Insulin treatment normalized tubular luminal area and glomerular tuft volume but did not completely reverse the increases in RPTC volume in Akita mice (**Table I**).

Masson's trichrome staining, TGF-β1 and collagen type IV immunostaining revealed enhanced expression of collagenous components (**Figure 2b**), TGF-β1 (**Figure 2d**) and collagen type IV (**Figure 2f**) in Akita mouse kidneys compared to WT controls. Once again, insulin treatment normalized these changes. Quantitative analysis of Masson trichrome-stained areas (**Figure 2c**), RT-qPCR for *TGF-β1* and *collagen IV* mRNA expression (**Figures 2e** and **2g**, respectively) confirmed these findings. These data indicate that insulin treatment effectively prevented tubulointerstitial fibrosis in Akita mice.

Agt, hnRNP F, hnRNP K, ACE and Ace2 Expression in Mouse Kidneys

We detected increased Agt immunostaining in RPTCs of Akita mice compared to WT controls, and this was normalized with insulin treatment (**Figure 3a**). In contrast, Akita mice RPTCs exhibited decreased immunostaining

for both hnRNP F (**Figure 3b**) and hnRNP K (**Figure 3c**) compared to WT controls. Insulin treatment led to higher than normal expression of hnRNP F and hnRNP K. Western blotting for Agt, hnRNP F and hnRNP K (**Figures 3d-f**, respectively) and RT-qPCR for *Agt*, *hnRNP F* and *hnRNP K* mRNA expression (**Figures 3g-i**, respectively) from isolated RPTs confirmed these findings.

Interestingly, neither liver *Agt* mRNA and protein levels nor serum Agt and Ang II levels differed significantly in the 3 groups of mice studied (Electronic Supplementary Material (ESM) **Figure 1)**.

Immunostaining for Ace2 was decreased in RPTCs of Akita mice compared with WT controls and insulin treatment normalized Ace2 immunostaining in RPTCs of Akita mice (**Figure 4a**). In contrast, RPTCs of Akita exhibited increased immunostaining for ACE compared with WT controls (**Figure 4b**). Insulin treatment, however, had no apparent effect on ACE immunostaining in RPTCs of Akita mice (**Figure 4b**). RT-qPCR for *Ace2* and *ACE* mRNA expression (**Figure 4c** and **4d**, respectively) in isolated RPTs confirmed these findings.

To investigate whether insulin could directly inhibit *Agt* and enhance *hnRNP F*, *hnRNP K* and *Ace2* gene expression in RPTs *in vivo*, we incubated freshly isolated mouse RPTs in the absence or presence of insulin for 16 h *ex vivo*. **Figure 4e** shows high glucose stimulation of *Agt* mRNA and inhibition of *hnRNP F*, *hnRNP K* and *Ace2* mRNA expression in RPTs of WT mice. Insulin treatment reversed these changes. Similar trends were also observed for the effect

of high glucose and insulin on *Agt*, *hnRNP F*, *hnRNP K* and *Ace2* mRNA expression in Akita RPTs (**Figure 4f**). These findings demonstrate that insulin directly inhibits *Agt* and stimulates *hnRNP F*, *hnRNP K* and *Ace2* mRNA expression in RPTs cultured *ex vivo*.

Effect of Insulin on Agt, hnRNP F and hnRNP K Gene Expression in Rat RPTCs in vitro

Consistent with our *in vivo* observations, high glucose (25 mmol/l D-glucose) stimulated *Agt* gene promoter activity in RPTCs compared to normal glucose (5 mmol/l D-glucose plus 20 mmol/l D-mannitol), which was inhibited by insulin in a dose- and time-dependent manner (**Figures 5a** and **5b**, respectively). PD 98059 and U0126, but not wortmannin and Ly 294,002, prevented insulin inhibition of *Agt* gene promoter activity (**Figure 5c**) and *Agt* mRNA expression in RPTCs (**Figure 5d)**. In contrast, RPTCs cultured in high glucose medium exhibited decreased *hnRNP F* (**Figure 5e**) and *hnRNP K* mRNA expression (**Figure 5f**) as compared to RPTCs cultured in normal glucose medium. Insulin treatment normalized both *hnRNP F* and *hnRNP K* expression (**Figures 5e and 5f**). PD 98059 and U0126, but not wortmannin and Ly 294,002, rendered RPTCs insensitive to insulin. Thus, insulin can directly inhibit *Agt* and enhance *hnRNP F* and *hnRNP K* gene expression in RPTCs in high-glucose medium signaling through the p44/p42 MAPK pathway.

Next, we investigated whether knock-down of hnRNP F or hnRNP K could prevent insulin inhibition of *Agt* gene expression at the transcriptional

level. Transfection of RPTCs with *hnRNP F* siRNA or *hnRNP K* siRNA reduced endogenous hnRNP F or hnRNP K protein expression in a concentration-dependent manner, respectively (**ESM Figures 2a and 2b**). Scrambled siRNA had no effect. Transfection with either *hnRNP F* siRNA or *hnRNP K* siRNA attenuated insulin inhibition of *Agt* mRNA expression in a concentration-dependent manner (**Figures 6a** and **6b)**. A combination of both siRNA of hnRNP F and siRNA of hnRNP K was more effective in attenuation of insulin inhibition of *Agt* mRNA expression than either siRNA of hnRNP F or siRNA of hnRNP K alone (**Figure 6c**). *Agt* gene promoter activity assays confirmed that *hnRNP F* siRNA and *hnRNP K* siRNA abolish the inhibitory effect of insulin on *Agt* gene promoter activity in RPTCs in high glucose medium (**Figures 6d** and **6e**). Again, a combination of hnRNP F and hnRNP K siRNA was more effective than either hnRNP F siRNA or hnRNP K siRNA alone (**Figure 6f**). These findings lends additional support to the notion that insulin inhibition of *Agt* gene transcription is mediated, at least in part, via up-regulation of both hnRNP F and hnRNP K expression in RPTCs *in vivo*.

Effects of hnRNP F and hnRNP K on CREB-mediated Agt Gene Expression in Rat RPTCs in vitro

Transfection of rat RPTCs with either pRSV-*hnRNP F* or pRSV-*hnRNP K* attenuated the stimulatory effect of pRSV-*CREB* on *Agt* gene promoter activity (**Figure 7a**), Agt mRNA (**Figure 7b**) and Agt expression (**Figure 7c**). A

combination of pRSV-*hnRNP F* and pRSV-*hnRNP K* appears to be more effective in inhibiting pRSV-*CREB*-stimulation of *Agt* gene promoter activity, Agt mRNA and Agt expression than pRSV-*hnRNP F* or pRSV-*hnRNP K* alone (**Figure 7a-c**).

DISCUSSION

The present report identifies a novel mechanism underlying insulin inhibition of renal *Agt* gene expression and subsequent prevention of hypertension and kidney injury in Akita mice via up-regulation of renal expression of hnRNP F and hnRNP K. Our observations suggest that cellular levels of hnRNP F and hnRNP K might play a critical role in regulating *Agt* gene expression and RAS activation *in vivo*.

We reported previously that overexpression of *hnRNP F* prevents systemic hypertension and inhibits renal *Agt* gene expression and RPTC hypertrophy in diabetic Akita *hnRNP F*-Tg mice [23]. However, the molecular regulation of *hnRNP F* gene expression has not been explored. Extending our previous observations, here we provide *in vivo* and *in vitro* evidence that insulin stimulates hnRNP F and hnRNP K expression, which is critical for inhibition of renal *Agt* gene expression and the antihypertensive and renoprotective actions in Akita mice.

A major limitation of the present studies is that our experimental design cannot differentiate a “glucose-lowering effect” from a “direct effect” of insulin on renal *Agt, hnRNP F* and *hnRNP K* expression *in vivo.* Future studies are needed to address this issue.

HnRNP F and hnRNP K are members of the pre-mRNA-binding protein family [30] and they regulate gene expression at both the transcriptional and post-transcriptional levels. Indeed, it has been noted that hnRNP F/K engage in alternative splicing of various genes [31-34] and associate with TATA-binding protein (TBP), RNA polymerase II (Poly II), nuclear cap-binding protein complex and various transcriptional factors [35-37]. The molecular mechanisms underlying hnRNP F/K regulation of gene transcription are still incompletely understood.

The Akita mouse is an autosomal dominant model of spontaneous type 1 diabetes in which the *insulin2* gene is mutated [38]. Like patients with type 1 diabetes, Akita mice develop hyperglycemia and systemic hypertension, leading to cardiac hypertrophy and left ventricular diastolic dysfunction, as well as glomerulosclerosis and enhanced oxidative stress in RPTs [17, 23, 39-43].

A novel observation in our study is that in addition to lowering blood glucose level, insulin treatment also prevents systemic hypertension, attenuates KW/BW and HW/BW ratios and normalizes the GFR, ACR, pro-fibrotic gene expression and tubulointerstitial fibrosis in Akita mice. It appears that insulin lowering of SBP is mediated, at least in part, via inhibition of intrarenal *Agt* gene expression and RAS activation. This notion is supported by our previous findings that Agt-Tg mice and Akita Agt-Tg mice specifically overexpressing Agt in their RPTCs develop hypertension, renal hypertrophy and tubulointerstitial fibrosis and that RAS blockade reverses these pathologies [13, 17]. These observations

imply that intrarenal *Agt* gene expression and RAS activation play critical role(s) in the development of hypertension, renal and heart injury.

We did not detect significant differences in liver Agt, serum Agt and serum Ang II levels among the 3 different groups of mice studied. In contrast, RPT *Agt* mRNA and protein levels, urinary Agt and Ang II levels were significantly higher in Akita mice than in WT controls. Insulin treatment normalized RPT *Agt* mRNA and protein expression and urinary Agt and Ang II levels. It is noteworthy that urinary Agt levels in insulin-treated Akita mice appeared to be slightly higher than in WT controls without reaching statistical significance. These observations highlight tissue-specific regulation of hepatic and renal *Agt* gene expression and indicate that urinary Agt derives predominantly from RPTCs and to a lesser degree from extra-renal source(s) in diabetic mice. Indeed, recent studies [44-46] have shown that Agt filtered through the glomerulus in non-diabetic animals constitutes only a small portion of Agt detected in the urine of non-diabetic animals.

We also detected significantly lower renal Ace2 expression in RPTCs of Akita mice, which can be normalized with insulin treatment. Consistent with these observations, our *ex vivo* studies employing freshly isolated RPTs from non-Akita and Akita mice showed insulin stimulation of *Ace2* mRNA expression in normal and high glucose. These data imply a novel role for insulin in up-regulation of intrarenal Ace2 expression in preventing hypertension and renal injury in diabetes.

Our studies with pharmacological inhibitors indicate that insulin action on *Agt*, *hnRNP F* and *K* gene transcription is mediated via the p44/42 MAPK signalling pathway, confirming our earlier report [18]. Insulin has previously been reported to stimulate hnRNP K phosphorylation via p44/42 MAPK signalling and to enhance its cytoplasmic translocation [47, 48]. Consistently, we also observed increased cytoplasmic and nuclear hnRNP F and K in RPTCs of Akita mice treated with insulin as well as in RPTCs treated by insulin *in vitro* (ESM Figure 3).

We have also shown that knock-down of hnRNP F and hnRNP K by their respective siRNAs prevented insulin inhibition of *Agt* gene transcription in RPTCs in high glucose medium. These findings clearly indicate involvement of hnRNP F/K in mediating insulin inhibition of *Agt* gene expression in the diabetic mouse kidney. Nevertheless, additional studies employing RPTC-specific *hnRNP F* and *K* knockout mice are needed to firmly establish this pathway.

The molecular mechanisms by which hnRNP F and hnRNP K inhibit *Agt* gene expression remain to be investigated. Overexpression of CREB augmented *Agt* gene transcription [25] and high glucose enhanced CREB expression in RPTCs [49]. We found that overexpression of hnRNP F and/or hnRNP K attenuated the stimulatory effect of CREB on Agt gene transcription. These data imply that the beneficial actions of hnRNP F and hnRNP K are mediated, at least in part, via competition with CREB for binding to TBP and RNA Poly II, subsequently attenuating *Agt* gene transcription. Additional studies are needed to confirm this mechanism.

In summary, our data demonstrate that hnRNP F and hnRNP K mediate insulin inhibition of renal *Agt* gene expression, prevention of hypertension, and amelioration of kidney and cardiac hypertrophy in type 1 diabetic mice. These observations imply that dysregulation of hnRNP F/K expression *in vivo* may directly alter activation of intrarenal RAS and, therefore, contribute to hypertension and renal injury in diabetes. Thus, hnRNP F/K may be potential targets in the treatment of hypertension and kidney injury in diabetes.

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JSDC is the guarantor of this work, had full access to all study data, and takes responsibility for data integrity and the accuracy of data analysis. SA and JSDC were principal investigators and were responsible for the study conception and design. JSDC drafted the manuscript, and reviewed/edited manuscript. SA, CSL, IC, AS and SLZ contributed to the *in vivo* and *ex vivo* experiments and collection of data. JGF and JRI contributed to discussion, and reviewed/edited manuscript. All authors were involved in analysis and interpretation of data and contributed to the critical revision of the manuscript. All authors provided final approval of the version to be published.

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This manuscript or any significant part of it is not under consideration for publication elsewhere. The data, however, were presented, in part, as a poster communication at the 44th Annual Meeting of the American Society of Nephrology, San Diego, CA, USA, October 30-November 4, 2012.

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Table I. Physiological Measurements

	WT	Akita	Akita + insulin
Systolic blood pressure (mm Hg)	109 ± 1	130 ± 5***	113 ± 5 ††
Blood glucose (mmol/l)	7.0 ± 0.7	30.8 ± 0.7***	15.4 ± 3.9 †††
Body weight (g)	29.9 ± 0.8	22.5 ± 0.3***	23.7 ± 0.3***
Kidney weight (g)	0.35 ± .01	0.47 ± 0.12***	0.32 ± 0.09 †††
Heart weight (g)	0.13 ± 0.04	0.17 ± 0.05***	0.13 ± 0.04 †††
Kidney weight/body weight ratio (mg/g)	12.05 ± 0.44	20.72 ± 0.58***	13.62 ± 0.35 †††
Heart weight/body weight ratio (mg/g)	4.66 ± 0.33	7.92 ± 0.14***	5.76 ± 0.20*, †††
Glomerular tuft volume (X $10^3 \mu m^3$)	133.2± 5.9	175.8 ± 6.9***	125.6 ± 2.7 †††
RPTC volume(X $10^3 \mu m^3$)	6.7± 0.4	9.7 ± 0.4***	8.1 ± 0.3*, ††
Tubular laminar area (μm^2)	46.7± 4.3	69.9 ± 3.2***	51.2 ± 4.1 ††

*p<0.05, **p<0.01 and ***p<0.005 vs. WT

†p<0.05, ††p<0.01 and †††p<0.005 vs. Akita

FIGURE LEGENDS

Figure 1: Insulin normalizes elevated blood glucose levels, lowers systolic blood pressure (SBP) and ameliorates kidney injury in male Akita mice. (**a**) Longitudinal changes in mean blood glucose levels with fasting for 4-5 hrs in male WT (○), Akita (▲) and Akita mice + insulin implants (■). (**b**) Longitudinal changes in mean SBP (measured 2 to 3 times per animal per week in the morning without fasting) in male WT (○), Akita (▲) and Akita mice + insulin implants (■). Baseline SBP was measured daily over a 5-day period before initiation of treatment. (**c**) Urinary albumin/creatinine ratio, (**d**) Glomerular filtration rate (GFR), (e) urinary Ang II and (f) urinary Agtlevels at week 16 in WT controls, Akita and Akita mice + insulin implants. Urinary Agt and Ang II levels were normalized with urinary creatinine levels. Values are means ± SEM, N=8 per group. *p<0.05; **p<0.01; ***p<0.005; N.S., not significant (WT vs. Akita). †p<0.05; ††p<0.01; N.S., not significant (Akita vs. Akita treated with insulin). WT controls (empty bars); Akita (solid bars) and Akita mice + insulin implant (light grey bars).

Figure 2: Insulin ameliorates glomerulotubular fibrosis and suppresses pro-fibrotic gene expression in Akita mice. (**a**) Periodic acid Schiff (PAS) staining in mouse kidneys of WT control littermate, Akita mouse and Akita mouse + insulin implant. Magnification X100 and X600. (**b**) Masson's trichrome staining of collagenous components in mouse kidneys: WT control mouse, Akita mouse, and Akita mouse + insulin implant. Magnification X600. (**c**) Semi-quantitative

analysis of Masson's trichrome staining in glomerulotubular areas of kidney sections from WT control mice, Akita mice and Akita mice + insulin implants at the age of 16 weeks. (**d**) Immunohistochemical staining for TGF-β1 and (**e**) RT-qPCR of *TGF-β1* mRNA expression in RPTs of WT controls, Akita and Akita mice + insulin implants. Magnification X 600. (**f**) Immunostaining for collagen type IV 1α and (**g**) RT-qPCR of *collagen type IV 1α* in RPTs of WT control mouse, Akita mouse, and Akita mouse + insulin implant. Magnification X 600. Values are means ± SEM, n=8. ***p<0.005; N.S., not significant. WT controls (empty bars), Akita (solid bars) and Akita mice + insulin implant (light grey bars).

Figure 3. Agt, hnRNP F and hnRNP K expression in mouse kidneys at the age of 16 weeks. Immunohistochemical staining for Agt (**a**), hnRNP F (**b**) and hnRNP K (**c**) in kidneys of WT control mouse, Akita mouse and Akita mouse + insulin implant, employing respective rabbit anti-Agt, anti-hnRNP F and anti-hnRNP K polyclonal antibodies. Magnification X 200. Western blotting analysis of Agt (**d**), hnRNP F (**e**) and hnRNP K (**f**) expression in RPTs from kidneys of WT controls, Akita and Akita mice + insulin implants. RT-qPCR of *Agt* (**g**), *hnRNP F* (**h**) and *hnRNP K* (**i**) mRNA expression in RPTs of WT controls, Akita and Akita mice + insulin implants. Values are means ± SEM, n=8 per group. **p<0.01; ***p<0.005; N.S., not significant.WT controls (empty bars); Akita (solid bars) and Akita mice + insulin implant (light grey bars).

Figure 4. Ace2 and ACE expression in mouse kidneys at the age of 16 weeks and the effect of insulin on *Agt*, *hnRNP F/K* and *Ace2* mRNA expression in RPTs *ex vivo*.Immunohistochemical staining for Ace2 (**a**) and ACE (**b**) in kidneys of WT control mouse, Akita mouse and Akita mouse + insulin implant, employing respective rabbit anti-Ace2 and anti-ACE polyclonal antibodies. Magnification X600. RT-qPCR of *Ace2* (**c**) and *ACE* (**d**) mRNA expression in RPTs of WT controls, Akita and Akita mice + insulin implants. RT-qPCR of *Agt*, *hnRNP F*, *hnRNP K* and *Ace2* mRNA expression in RPTs isolated from WT control mice (**e**) and Akita mice (**f**). Freshly isolated RPTs were incubated in normal glucose (5 mmol/l D-glucose plus 20 mmol/l D-mannitol) or high glucose (25 mmol/l D-glucose) medium in the absence or presence of insulin (10^{-7}M or 100 mmol/l) for 16 hrs and then harvested for quantitation of mRNA expression. The levels of mRNA expression in cells incubated in medium containing 5 mmol/l D-glucose plus 20 mmol/l D-mannitol are expressed as 100 percentage of control. The effect of high glucose ± insulin is compared with cells cultured in normal glucose (5 mmol/l D-glucose plus 20 mmol/l D-mannitol. Values are means ± SEM, n=3 per group. *p<0.05; **p<0.01; ***p<0.005; N.S., not significant.Normal glucose (empty bars); normal glucose + insulin (light grey bars); high glucose (solid bars) and high glucose + insulin (dark grey bars).

Figure 5. Effect of insulin on *Agt* gene promoter activity, *Agt* and *hnRNP F/K* mRNA expression in rat RPTCs. Cells stably transfected with pGL4 *rAgt N-1498/+18* were incubated in normal glucose (5 mmol/l D-glucose plus 20

mmol/l D-mannitol or high glucose (25 mmol/l D-glucose) DMEM in the absence or presence of various concentrations of insulin for 24 h (**a**) or for various time periods (**b**) with or without PD 98059 (10^{-5} M), U0126 (10^{-6} M), wortmannin (10^{-6} M) or Ly 294,002 (10^{-6} M) (**c**). Cells were harvested and assayed for luciferase activity. In **b**, normal glucose (▲); normal glucose + insulin (10^{-7} M or 100 mmol/l) (□); high glucose (■); and high glucose + insulin (10^{-7} M or 100 mmol/l) (Δ). Effect of insulin on *Agt* mRNA (**d**), *hnRNP F* mRNA (**e**) and *hnRNP K* mRNA (**f**) expression in high glucose medium in the absence or presence of PD 98059, U0126, wortmannin or Ly 294,002. The levels of luciferase activity or mRNA in cells incubated in medium containing 5 mmol/l D-glucose plus 20 mmol/l D-mannitol are expressed as 100 percentage of control. The inhibitory effect of insulin is compared with cells cultured in 25 mmol/l D-glucose only. The results are expressed as percentages of control values (mean ± SEM, n=3). **$p \leq 0.01$; ***$p \leq 0.005$. Similar results were obtained in 2 separate experiments.

Figure 6. Effect of *hnRNP F* and *hnRNP K* siRNA on insulin inhibition of *Agt* gene expression in RPTCs. (**a**) Effect of *hnRNP F* siRNA on *Agt* mRNA expression in RPTCs incubated in normal or high glucose medium ± insulin quantified by RT-qPCR. (**b**) Effect of *hnRNP K* siRNA on *Agt* mRNA expression in RPTCs incubated in normal or high glucose medium ± insulin quantified by RT-qPCR. (**c**) Effect of a combination of *hnRNP F and hnRNP K* siRNA on *Agt* mRNA expression in RPTCs incubated in normal or high glucose medium ±

insulin quantified by RT-qPCR. The levels of *Agt* mRNA in cells incubated in normal glucose medium are expressed as arbitrary unit 1. The inhibitory effect of insulin was compared with cells cultured in 25 mmol/l D-glucose. (**d**) Effect of *hnRNP F* siRNA or scrambled siRNA on *Agt* gene promoter activity in RPTCs cultured in normal or high glucose medium ± insulin. (**e**) Effects of *hnRNP K* siRNA or scrambled siRNA on *Agt* gene promoter activity in RPTCs cultured in normal or high glucose medium ± insulin. (**f**) Effect of a combination of *hnRNP F and hnRNP K* siRNA on *Agt* gene promoter activity in RPTCs incubated in normal or high glucose medium ± insulin quantified by RT-qPCR. Cells were harvested following 24 h of incubation, and *Agt* gene promoter activity was assessed by the luciferase activity assay. The results are expressed as percentages of control values (mean ± SEM, n=3). *p<0.05; **p<0.01; ***p≤ 0.005. N.S., not significant. Sc, scrambled.

Figure 7. Effect of *hnRNP F*, *hnRNP K* and *CREB* on *Agt* gene promoter activity,*Agt* mRNA and Agt protein expression in RPTCs. 48 h after co-transfection of pRSV or pRSV-*CREB* with or without pRSV-*hnRNP F*, pRSV-*hnRNP K* into cells stably transfected with pGL4-*Agt N-1498/+18* or RPTCs cultured in 5 mM D-glucose medium, the cells were harvested and assayed for luciferase activity (**a**) or *Agt* mRNA (**b**) or Agt protein (**c**) levels. Relative luciferase activity or *Agt* mRNA or Agt protein levels in cells transfected with 4.0 ug of pRSV was considered as 100% (control). Each point represents the mean ± SEM (n=3; assayed in duplicate). *p<0.05; **p<0.01; ***p≤ 0.005. N.S., not significant.

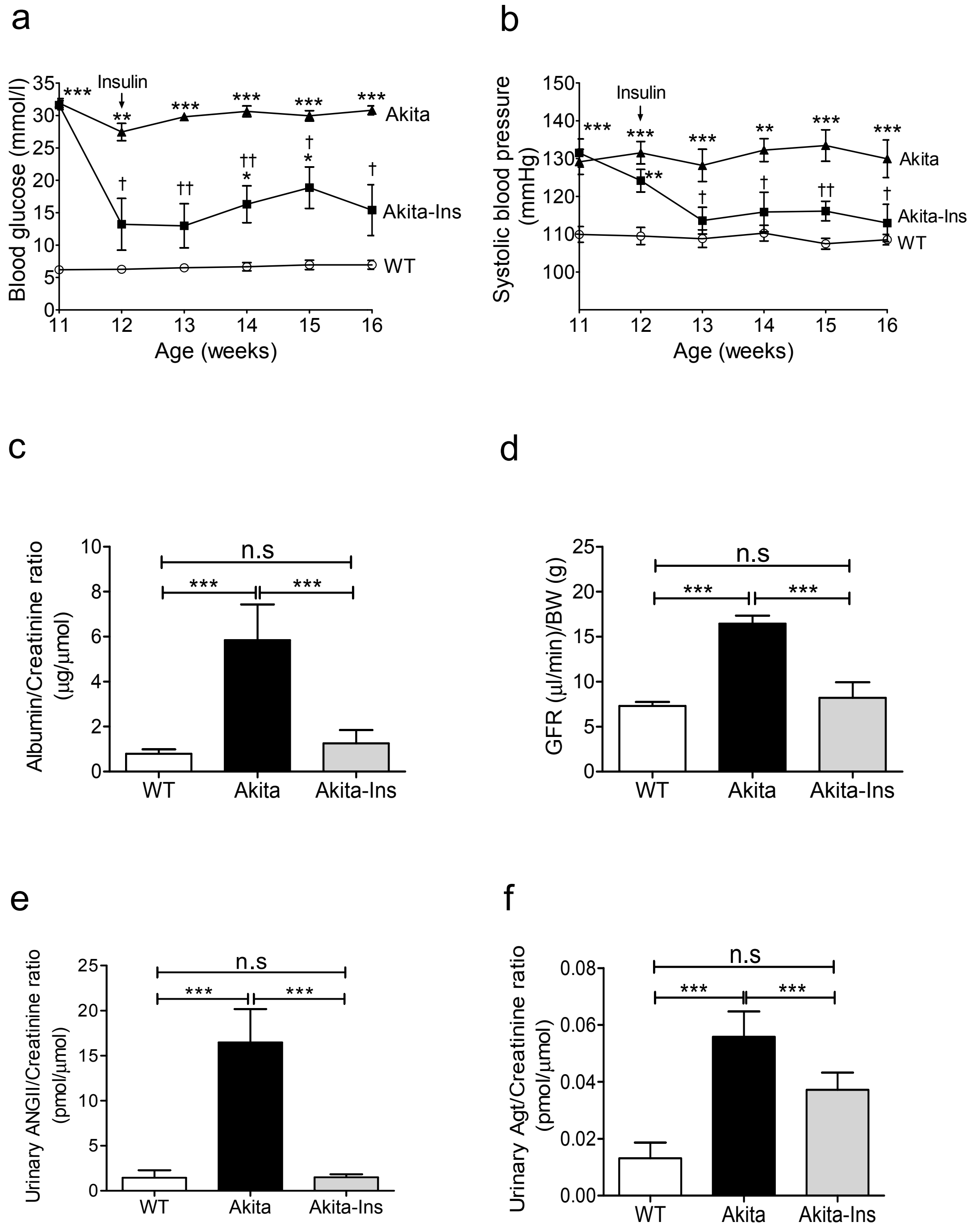


Figure 1

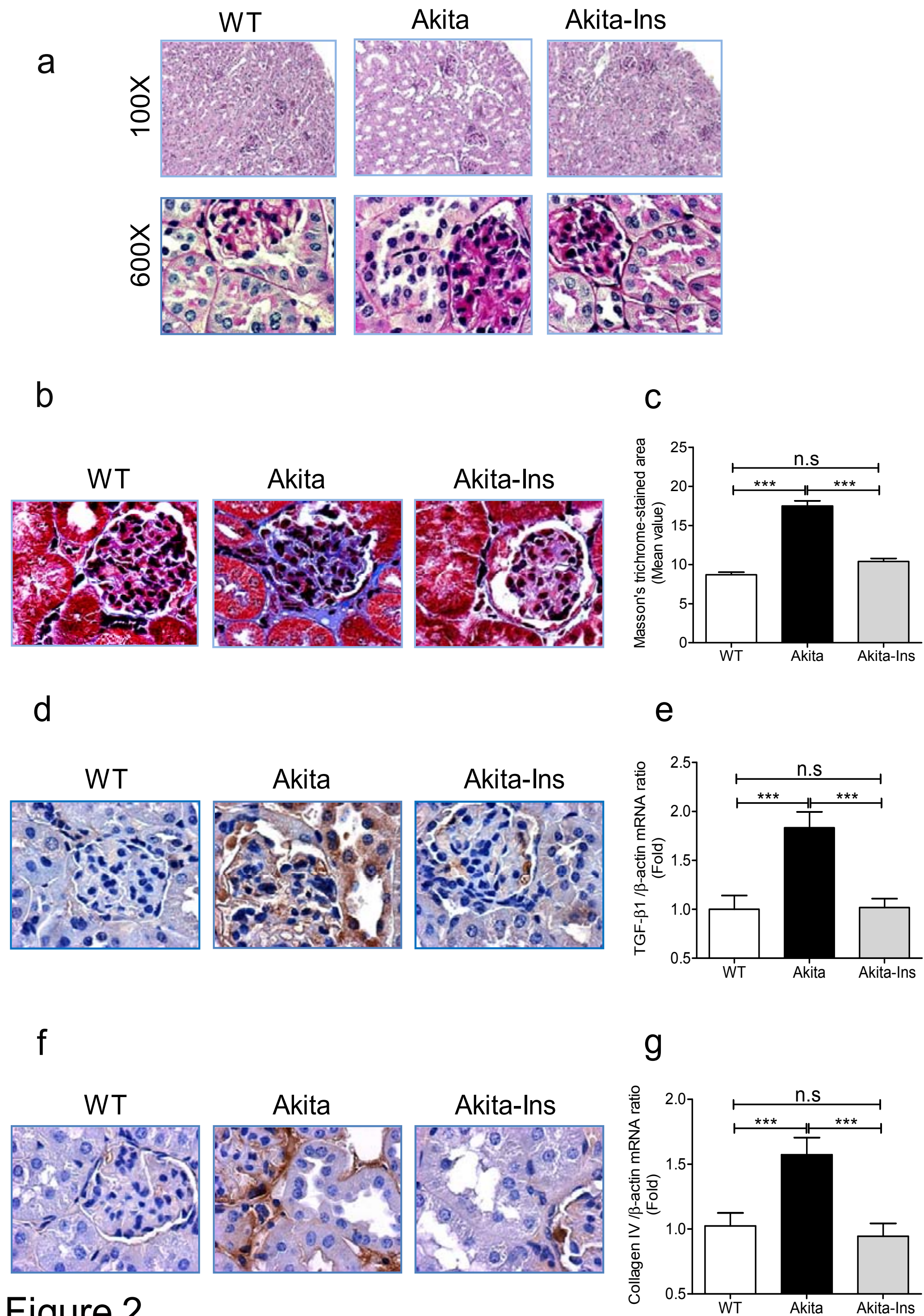
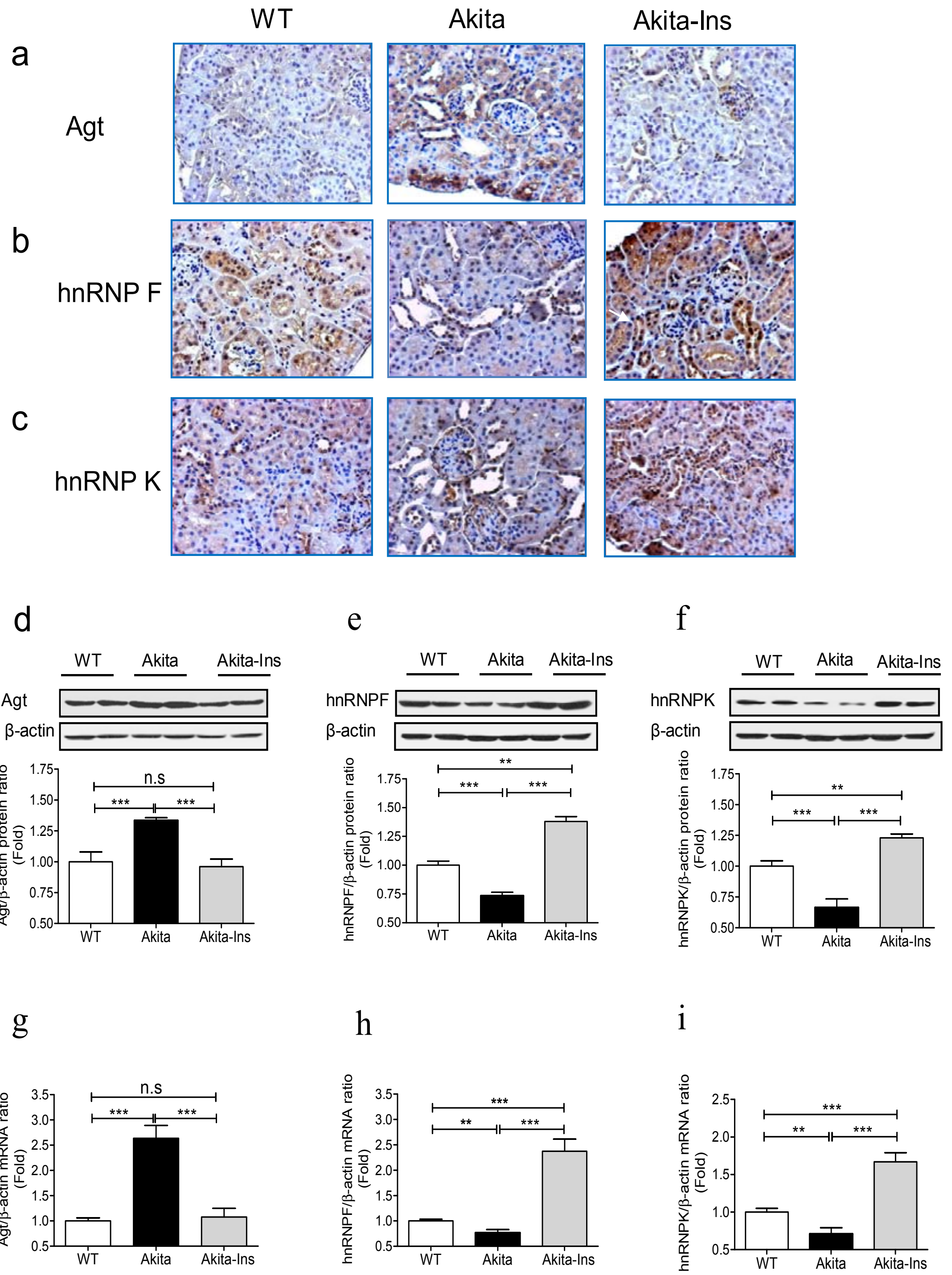


Figure 2



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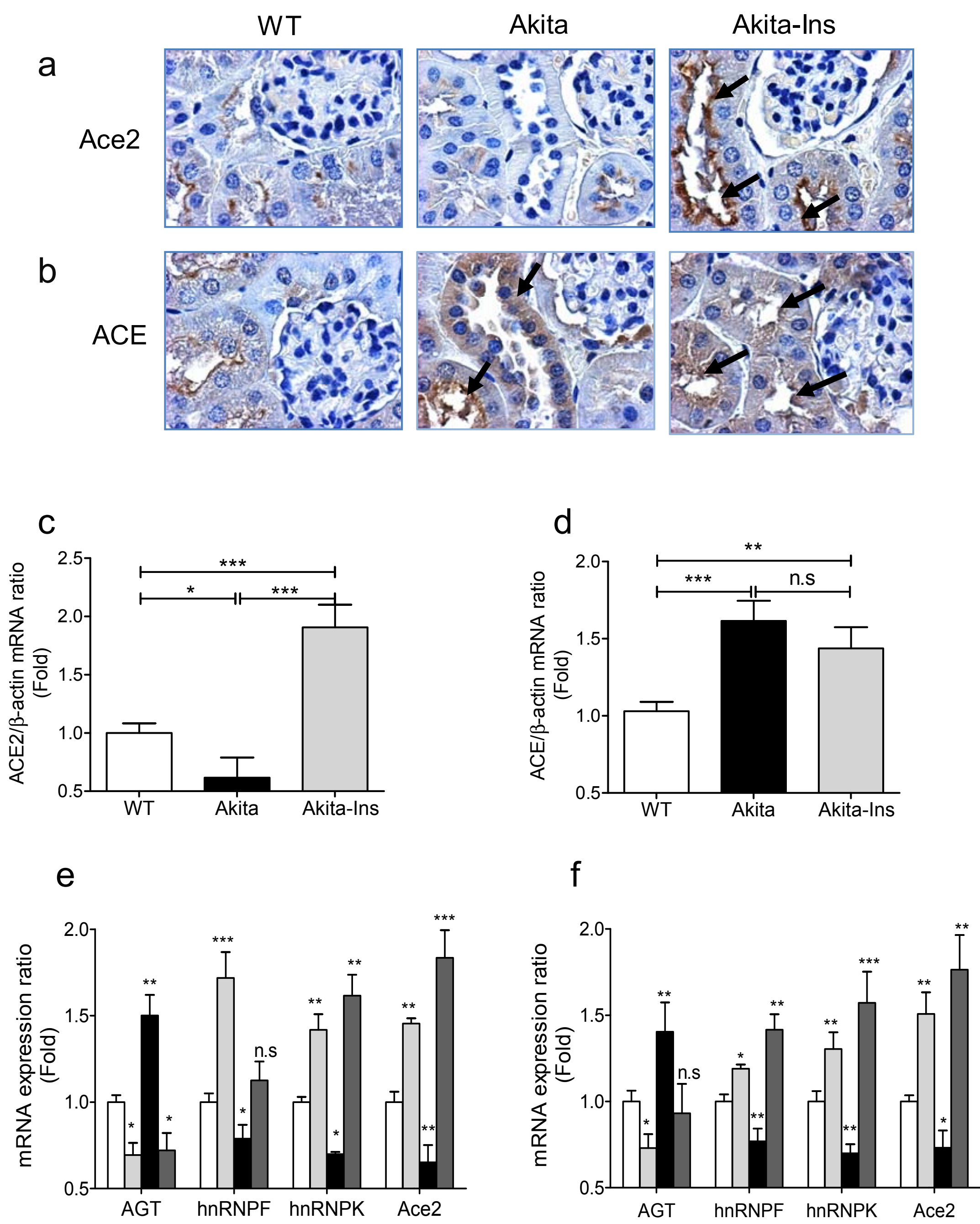
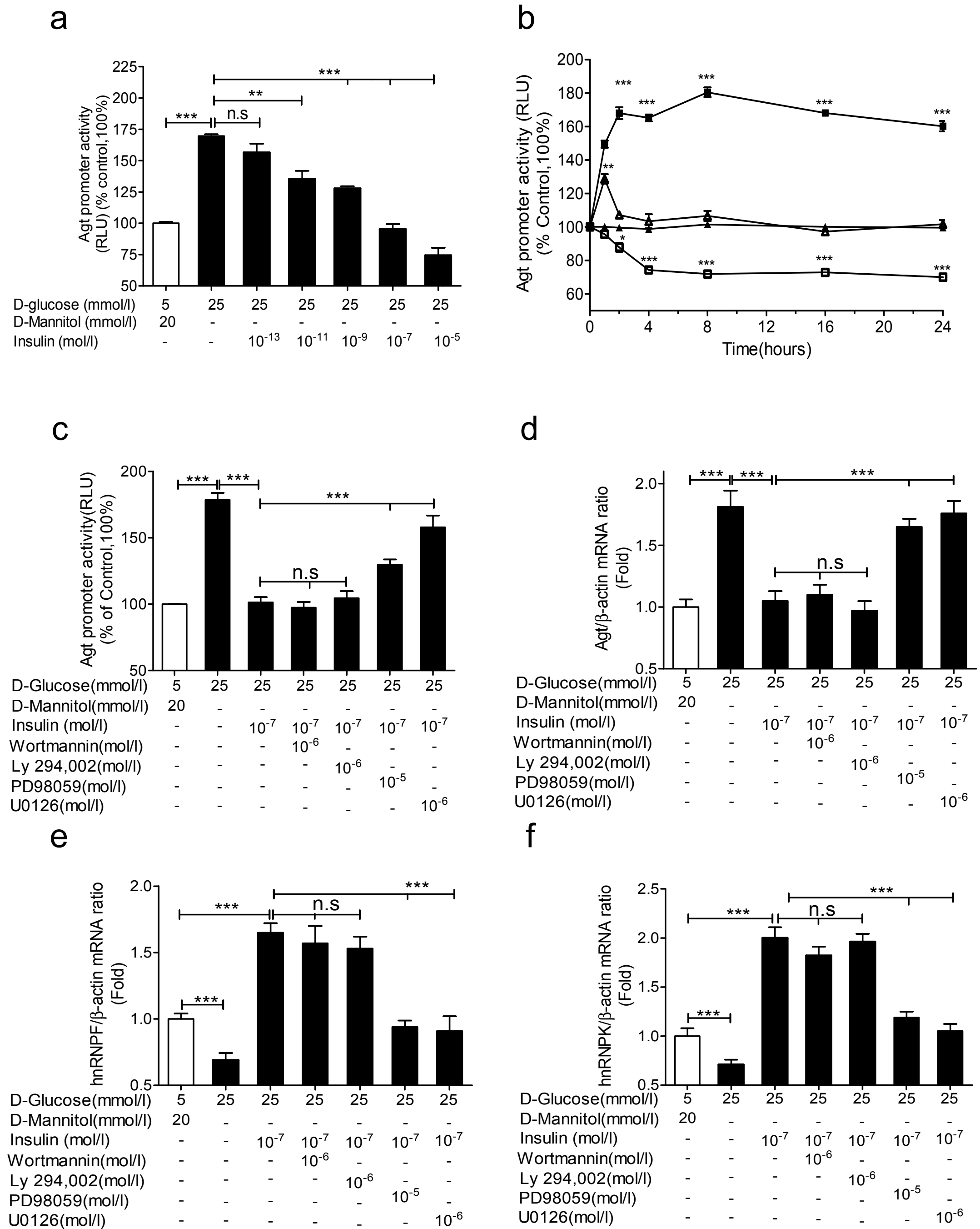


Figure 4



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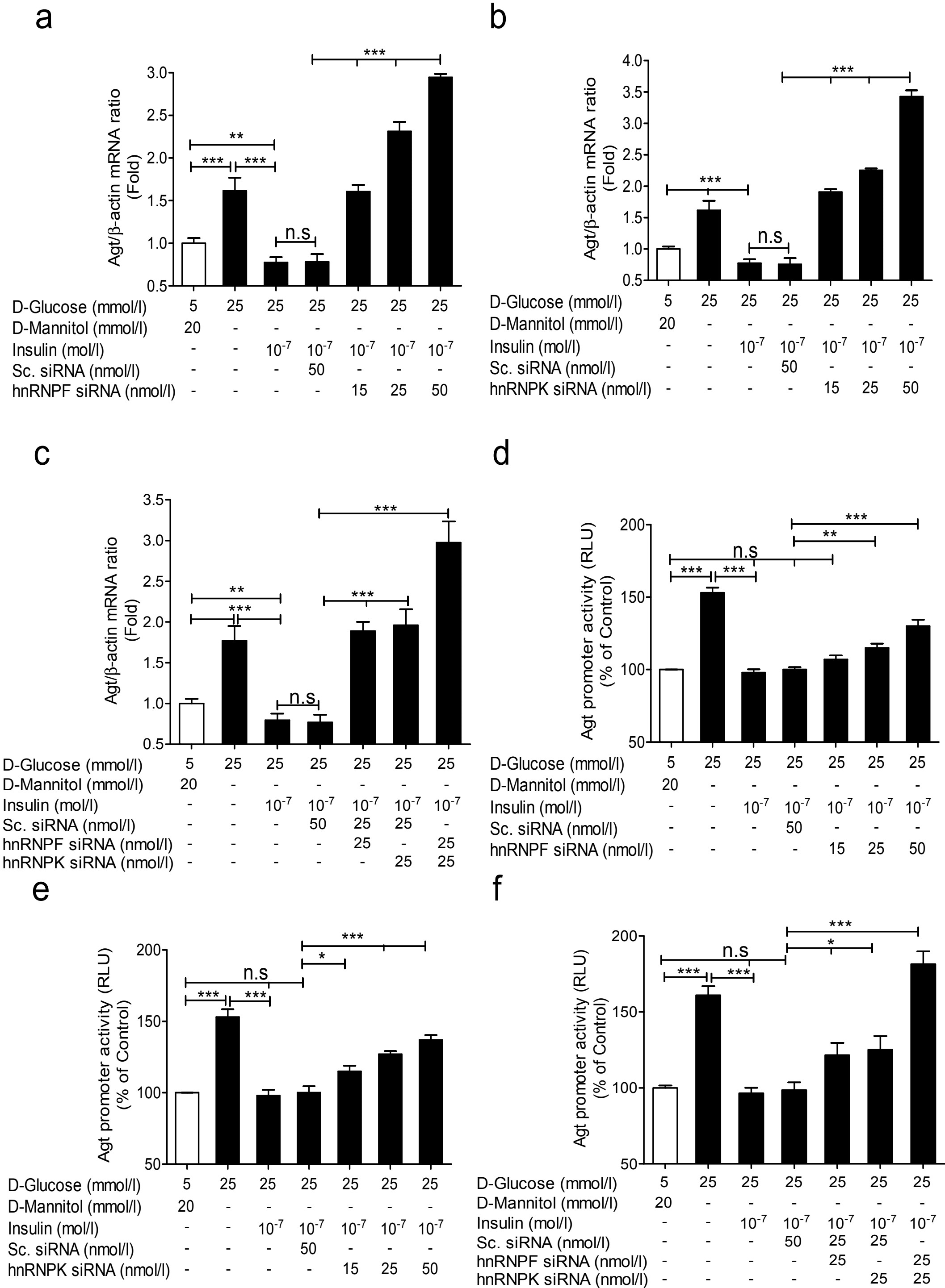
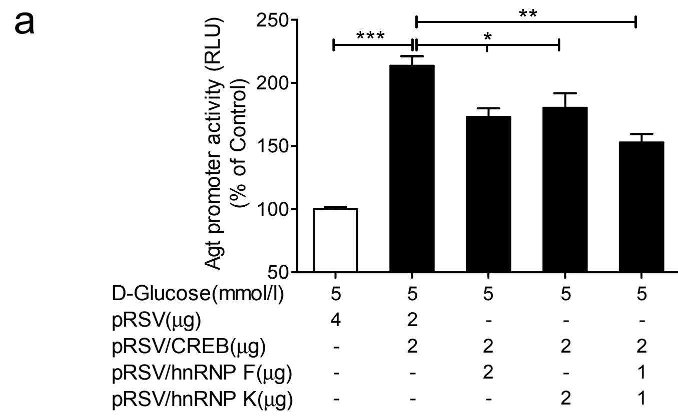
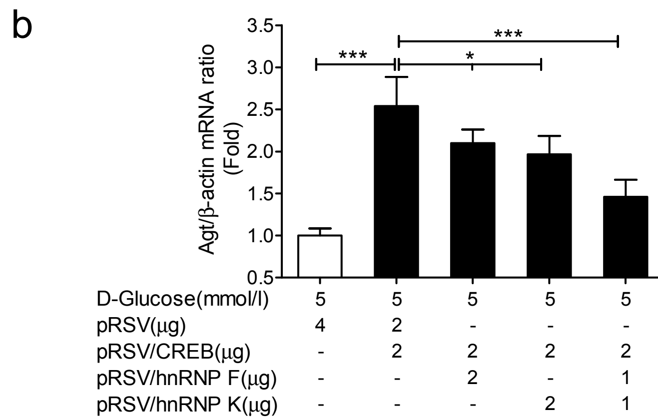


Figure 6





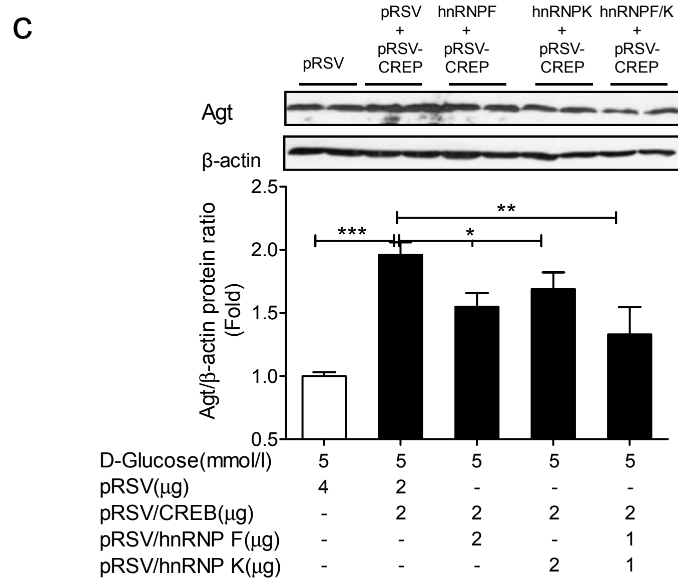
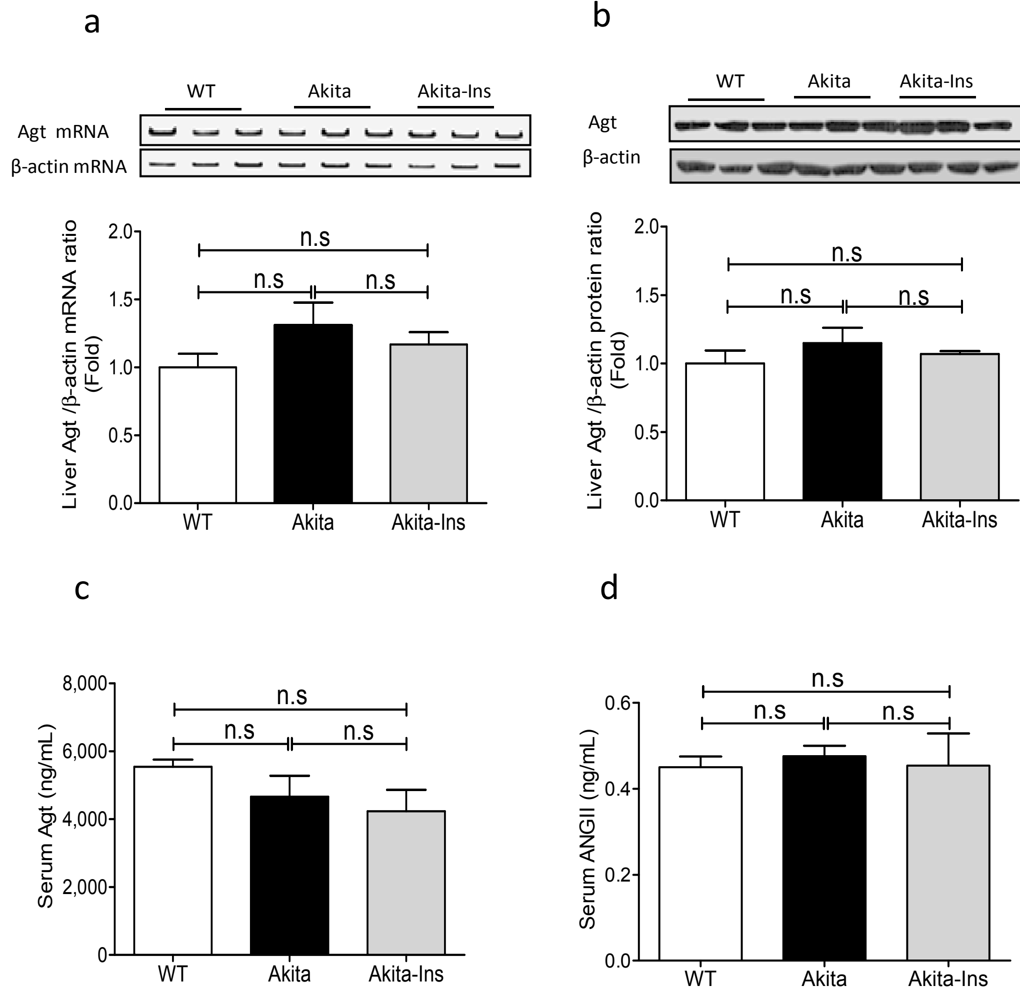


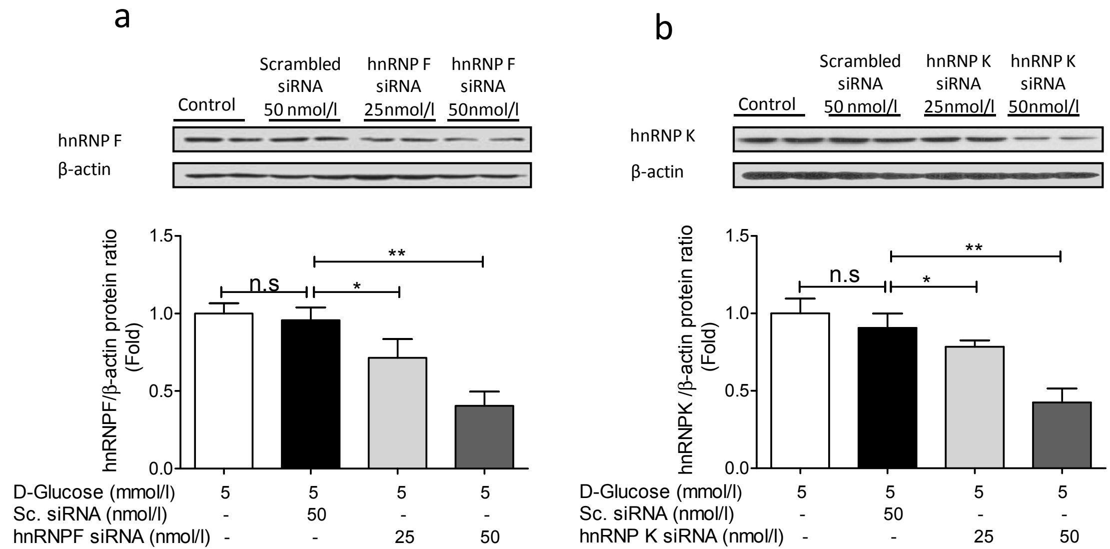
Figure 7

Electronic Supplementary Materials (ESM) Figure 1



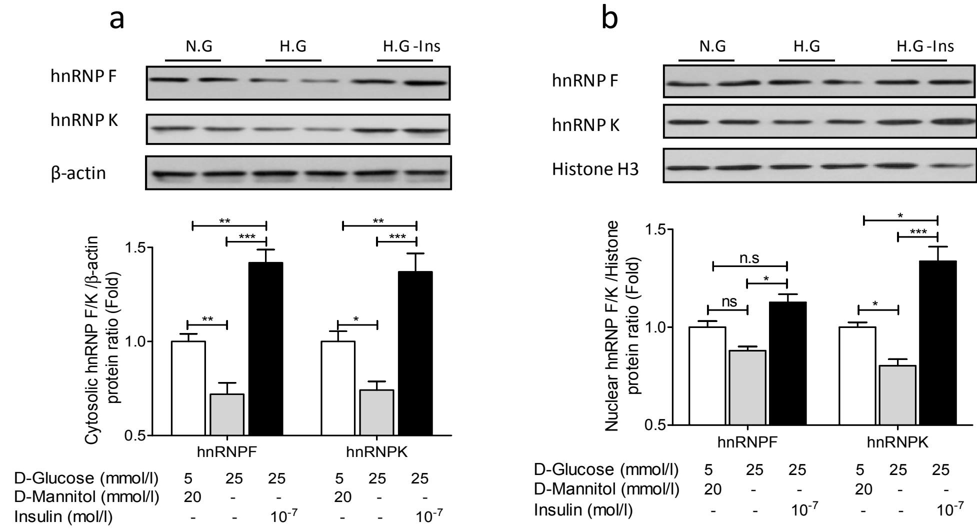
Effect of insulin on liver Agt expression, serum Agt and Ang II levels in Akita mice.
a. *Agt* mRNA, b. Agt protein, c. Serum Agt levels, and d. Serum Ang II levels in WT controls, Akita and Akita mice + insulin implants. Liver *Agt* mRNA levels were quantified by RT-qPCR. Agt protein levels in liver extracts were analyzed by Western blotting whereas serum Agt and Ang II samples were assayed by specific ELISA. Values are means ± SEM, n=8 per group. *p<0.05; **p<0.01; N.S., not significant. WT controls (empty bars); Akita (solid bars) and Akita mice + insulin implant (light grey bars).

ESM Figure 2



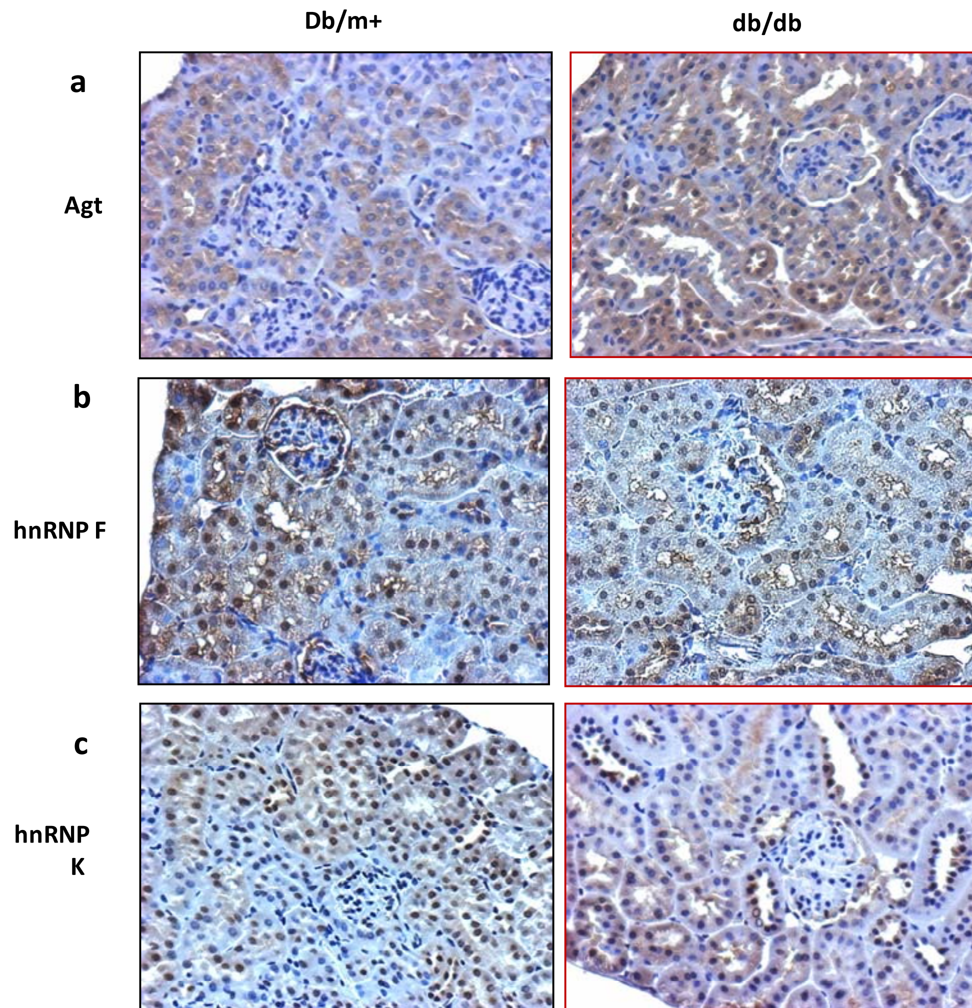
Effect of *hnRNP F* and *hnRNP K* siRNA on respective hnRNP F and hnRNP K protein expression in RPTCs. a. Western blotting of hnRNP F in RPTCs incubated in 5 mmol/l D-glucose medium with increasing doses of either *hnRNP F* siRNA or scrambled siRNA. b. Western blotting of hnRNP K in RPTCs incubated in 5 mmol/l D-glucose medium with increasing doses of either *hnRNP K* siRNA or scrambled siRNA. Values are mean ± SEM, n=3. *$p<0.05$; **$p<0.01$; N.S., not significant. Sc, scrambled.

ESM Figure 3



Effect of insulin on cytosolic and nuclear hnRNP F and hnRNP K expression in RPTCs in vitro. a. Western blotting of cytosolic hnRNP F and hnRNP K expression and b. Western blotting of nuclear hnRNP F and hnRNP K expression in RPTCs cultured in 5 mM D-Glucose + 20 mM D-Mannitol (empty bar), 25 mM D-Glucose (light grey bar), and 25 mM D-Glucose + Insulin (10^{-7} mol/l) (solid bar). Cells were harvested and fractionated into cytosolic and nuclear fraction by Nuclear Isolation Kit (Cytosol/Nuclear Fractionation Kit, Enzo Life Sciences, Farmingdale, NY, USA). Values are means ± SEM, n=3 per group. *$p<0.05$; **$p<0.01$; ***$p<0.005$; N.S., not significant.

ESM Figure 4



Agt, hnRNP F and hnRNP K expression in db/m+ and db/db mouse kidneys at the age of 16 weeks. Immunohistochemical staining for a. Agt, b. hnRNP F, and c. hnRNP K in kidneys of non-diabetic db/m+ mouse and diabetic db/db mouse, employing respective rabbit anti-Agt, anti-hnRNP F and anti-hnRNP K polyclonal antibodies. Magnification X 200.

Chapter 3 : Article 2

Catalase Overexpression Prevents Nuclear Factor Erythroid 2-Related Factor 2 Stimulation of Renal Angiotensinogen Gene Expression, Hypertension and Kidney Injury in Diabetic Mice.

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Catalase Overexpression Prevents Nuclear Factor Erythroid 2-Related Factor 2 Stimulation of Renal Angiotensinogen Gene Expression, Hypertension and Kidney Injury in Diabetic Mice

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Running title: Nuclear Factor Erythroid 2-Related Factor 2and Diabetic Kidney Injury

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Subject categories: Pathophysiology of renal disease and progression

Abstract: 200 words; Text: 3,986

ABSTRACT

This study investigated the impact of catalase (Cat) overexpression in renal proximal tubule cells (RPTCs) on nuclear factor erythroid 2-related factor 2 (Nrf2) stimulation of angiotensinogen (Agt) gene expression and development of hypertension and renal injury indiabeticAkita transgenic (Tg) mice. Additionally, adult male mice were treated with the Nrf2 activator oltipraz ± an inhibitor trigonelline. Rat RPTCs, stably transfected with plasmid containing either rat Agt or Nrf2 gene promoter, were also studied. Cat overexpression normalized systolic BP, attenuated renal injury, and inhibited RPTC Nrf2, Agt and heme oxygenase-1 (HO-1) gene expression in Akita Cat-Tg compared to Akita mice. In vitro, high glucose, hydrogen peroxide and oltipraz stimulated Nrf2 and Agt gene expression; these changes were blocked by trigonelline, small interfering RNA of Nrf2, antioxidants, or pharmacological inhibitors of NF-kB and p38 mitogen-activated protein kinase. Deletion of Nrf2-responsive elements in the rat Agt gene promoter abolished the stimulatory effect of oltipraz. Oltipraz administration also augmented Agt, HO-1 and Nrf2 gene expression in mouse RPTCs, and was reversed by trigonelline.These data identify a novel mechanism, Nrf2-mediated stimulation of intrarenal Agt gene expression and activation of the renin-angiotensin system, by whichhyperglycemia induces hypertension and renal injury in diabetic mice.

INTRODUCTION

Hyperglycemia, oxidative stress and dysregulation of the renin-angiotensin system (RAS) have long been implicated in the development and progression of diabetic nephropathy. However, the underlying molecular mechanisms remain incompletely understood. In addition to the systemic RAS, the existence of a local intrarenal RAS in renal proximal tubular cells (RPTCs) has been well-documented (1). Several lines of evidence indicate that enhanced generation of reactive oxygen species (ROS) is central to the development of hypertension and RPTC apoptosis in diabetes. ROS mediate high-glucose (HG) stimulation of angiotensinogen (Agt, the sole precursor of all angiotensins) gene expression in RPTCs *in vitro* (2-5).Transgenic (Tg) mice specifically overexpressing rat (r) Agt in their RPTCs develop hypertension and kidney injury (6).Hyperglycemia and Agt overexpression act in concert to induce hypertension and RPTC apoptosis in diabetic Agt-Tg mice (7, 8). Conversely, apocynin treatment (9)and catalase (Cat) overexpression attenuate hypertension and RPTC apoptosis in non-diabetic Agt/Cat-Tg (10)and diabetic Cat-Tgmice (11-13).

Nuclear factor erythroid 2-related factor 2 (Nrf2) functions as a master regulator of redox balance in cellular cytoprotective responses (14). Nrf2 is normally sequestered in the cytoplasm by a cytosolic repressor, Keap1 (Kelch-like ECH-associated protein 1) and is constantly degraded (15). However, with oxidative stress, Nrf2 is released from Keap1

repression, translocates to the nucleus, forms heterodimers with small musculoaponeurotic fibrosarcoma proteins, binds to antioxidant-response elements (AREs) and initiates transcription of an array of genes (16). Little information is available about the functional relationship between ROS, *Nrf2* and *Agt* gene expression in diabetic RPTCs, which may be critical for development of diabetic renal injury.

In the present study, we investigated the relation between oxidative stress,*Nrf2* and *Agt* gene expression, hypertension development and RPTC injury in HG milieu both *invivo* and *in vitro*. We report that Cat overexpression prevented hyperglycemia-induced stimulation of *Nrf2, HO-1* and *Agt* gene expression in RPTCs, subsequently attenuating hypertension and ameliorating renal injury in diabetic Akita mice. *In vitro*, HG, H_2O_2 and the Nrf2 activator oltipraz stimulated *Nrf2, HO-1* and *Agt*gene expression in RPTCs, which can be reversed by trigonelline (a Nrf2 inhibitor), small interfering RNAs (siRNAs) of Nrf2, antioxidants, and pharmacological blockade of ***p38 mitogen-activated protein kinase***(p38 MAPK) and nuclear factor kappa-light-chain-enhancer of activated B cell (NF-κB) signaling. Consistently, *in vivo* administration of oltipraz stimulated *Nrf2, HO-1* and *Agt* gene expression in mouse renal proximal tubules (RPTs), which was reversed by trigonelline co-administration.

MATERIALS AND METHODS

Chemicals and Constructs

D-glucose, D-mannitol, hydrogen peroxide (H_2O_2), oltipraz (a Nrf2 activator), the alkaloid trigonelline (***C7H7NO2, a Nrf2 inhibitor),*** PD98059 (a p44/42 MAPK inhibitor), wortmannin (an inhibitor of phosphatidylinositol 3-kinase (PI3-K)) and anti-β-actin monoclonal antibody were purchased from Sigma-AldrichCanada Ltd. (Oakville, ON, Canada). SB203580 (an inhibitor of p38 MAPK) was obtained from Cell Signaling Technology (New England Biolabs Ltd., Whitby, ON, CA). PDTC (pyrrolidinedithiocarbamate ammonium)and BAY-11-7082(inhibitors of NF-κB activation) were from Calbiochem (San Diego, CA, USA). Normal glucose (5 mmol/l D-glucose) Dulbecco's Modified Eagle's Medium (DMEM, Catalogue No. 12320), penicillin/streptomycin and fetal bovine serum (FBS) were procured from Invitrogen, Inc. (Burlington, ON, Canada). Anti-Nrf2and anti-Keap1 antibodies were obtained from BD Biosciences (Mississauga, ON, Canada) and R & D Systems, Inc. (Minneapolis, MN, USA), respectively. Polyclonal anti-HO-1 antibodies were purchased from Assay Designs (Ann Arbor, MI, USA). Rabbit polyclonal antibodies specific for *rAgt*(17) were generated in our laboratory (J.S.D.C). The plasmid pKAP2 containing the kidney-specific androgen-regulated protein (KAP) promoter that is responsive to androgen was a gift from Dr. Curt D. Sigmund (University of Iowa, Iowa, IA, USA) (18). The plasmid pcDNA3.1 containing the Flag-(RelA)

p65cDNAwas a gift from Dr. Marc Servant (Faculty of Pharmacy, Université de Montréal, Montreal, QC, Canada). Full-length r*Cat* cDNA fused with HA-tag (which encodes amino acid residues 98-106 of human influenza virus hemagglutinin at the carboxyl terminal with the *NotI* site at both 5'- and 3'-termini) was inserted into plasmid pKAP2 at the *NotI* site (11). The *rAgt* gene promoter (N-1495 to N+18) (19) and the rat *Nrf2* gene promoter (N-1980 to N+111) (20) were cloned from respective rat genomic DNA by conventional polymerase chain reaction (PCR) with specific primers and inserted into pGL4.20 vector via Kpn I and Hind III restriction sites (19). Quick Change Site-Directed Mutagenesis kits from Stratagene Inc. (La Jolla, CA) were used to delete putative *Nrf2*-REs in the r*Agt* gene promoter (21). **Table 1** details the oligo primers for cloning of the *rAgt* and r*Nrf2* gene promoters and site-directed mutagenesis. Scrambled Silencer Negative Control #1 siRNA and *Nrf2* siRNA were obtained from Ambion, Inc. (Austin, TX, USA). Oligonucleotides were synthesized by Invitrogen, Inc. Restriction and modifying enzymes were obtained from commercial sources. Viable and fertile mice heterozygous for the Akita spontaneous mutation of insulin 2 (*Ins2*) gene (C57BL/6-*Ins2*^{Akita}/J) were purchased from Jackson Laboratories, Bar Harbor, ME, USA (http://jaxmice.jax.org).

Generation of Akita Cat-Tg Mice

We generated Tg mice specifically overexpressing Cat in their

RPTCs by cross-breeding homozygous Cat-Tg mice (11) with heterozygous Akita mice (N.B.: Homozygous Akita mice are infertile).These mice are homozygous for the *Cat* transgene but heterozygous for *Ins2* gene mutation(8, 13).

Pathophysiological Studies

Male adult (16-week-old) non-Akita wild type (WT), Cat-Tg, Akita and Akita Cat-Tg mice (8 per group) were used. All animals received standard mouse chow and water *ad libitum*. Animal care and experimental procedures were approved by the CRCHUM Animal Care Committee.

Systolic blood pressure (SBP) was monitored with a BP-2000 tail-cuff pressure machine (Visitech Systems, Apex, NC, USA) in the morning,at least 2-3 times a week, for 5 weeks (6-13, 19).

The glomerular filtration rate (GFR) was estimated according to the protocol described by Qi et al. (22), as recommended by the Animal Models of Diabetic Complications Consortium (http://www.diacomp.org/) (13, 19).

Body weight (BW) was recorded 24 h prior to euthanasia, and the mice were housed individually in metabolic cages. Blood (~500 to 1,000 µl) was collected from each mouse by intra-cardiac puncture before euthanasia and centrifuged for serum. Urine (~100 to 400 µl/mouse) was

analyzed by albumin enzyme-linked immunosorbent assay (ELISA, Albuwell, Exocell, Inc., Philadelphia, PA, USA) and creatinine kit (Creatinine Companion, Exocell, Inc.) (6-13, 19).

After euthanization, the kidneys were removed, decapsulated and weighed. Left kidneys were processed for histology and immunostaining, and right kidneys were harvested for isolation of RPTs by Percoll gradient (6-13, 19). Aliquots of freshly-isolated RPTs from individual mice were immediately processed for total RNA or protein analysis.

In separate experiments, adult male non-Akita WT mice (age 14 weeks) received an injection of either corn oil (5 $ml.kg^{-1}$ of BW) or oltipraz (150 $mg.kg^{-1}day^{-1}$, *i.p.* in corn oil) ± trigonelline (0.02 $mg.kg^{-1}.day^{-1}$, *i.p.* in 0.9% NaCl) 4 times every other day (i.e., on Days 1, 3, 5 and 7) according to published protocols (8 mice per group) (23, 24). Animals were euthanized 24 h after the last injection.

Histology

Tissue sections (4-5 sections per kidney) were counterstained with periodic acid Schiff and analyzed by light microscopy by 2 investigators blinded to the treatments.

Immunostaining was performed with the standard avidin-biotin-peroxidase complex method on 4 to 5 sections per kidney and 3 mouse kidneys per group (ABC Staining; Santa Cruz Biotechnology, Santa Cruz, CA, USA) (6-13, 19).

Oxidative stress in RPTs was assessed by dihydroethidium (DHE; Sigma) staining in frozen kidney sections (13). The results were confirmed by standard assays of Cat activity (11), ROS generation (4, 5, 10-12), NADPH oxidase activity (10) and *Nox4* mRNA expression (25).

Real Time-quantitative Polymerase Chain Reaction (RT-qPCR)

Agt, Nrf2, Keap1, HO-1 and *β-actin* mRNA expression in RPTs was quantified by RT-qPCR with forward and reverse primers (**Table 1)** (7-13, 19).

Western Blotting

Western Blotting (WB) was performed as described previously (6-13, 19). The relative densities of Agt, Nrf2, Keap1, HO-1 and β-actin bands were quantified by densitometry using ImageQuant software (version 5.1, Molecular Dynamics, Sunnyvale, CA, USA).

Serum and Urinary Agt and Angiotensin II (Ang II) Measurement

Serum and urinary Agt were quantified by ELISA (Immuno-Biological Laboratories Inc. Minneapolis, MN, USA) (8, 10, 13, 19). To measure Ang II levels, serum and urine samples were extracted using a kit and were assayed by specific ELISA for Ang II (Bachem Americas Inc., Torrance, CA, USA) (8, 10, 13, 19).

Effect of HG, H_2O_2 and Oltipraz on Agt and Nrf2 Gene Expression in rRPTCs

Immortalized rRPTCs (passages 12 through 18) (26) were studied. The plasmids pGL4.20-r*Agt N-1495/+18* and pGL4.20-r*Nrf2 N-1980/+111* were stably transfected into rRPTCs (designated as stable transformants) (19).

To study the effects of HG, H_2O_2 and oltipraz, rRPTCs at 75-85% confluency or stable transformants were synchronized overnight in serum-free DMEM containing 5 mmol/l D-glucose, then incubated in medium containing 5 mmol/l D-glucose plus H_2O_2 (10^{-6} M) or (5 mmol/l D-glucose plus 20 mmol/l D-mannitol) (normal glucose) in the absence or presence of oltipraz ± trigonelline or HG (25 mmol/l D-glucose) DMEM containing 1% depleted FBS for 24 h in the presence of antioxidants, NF-κB inhibitors (PDTC or BAY-11-7082), the p38 MAPK inhibitor (SB203580), the p44/42 MAPK inhibitor (PD98059), or wortmannin (a PI3-K inhibitor) (2-5) [115, 228, 414] [115, 228, 414]. *Agt, HO-1* and *Nrf2* mRNA levels were quantified by RT-qPCR, and corresponding *Agt* and *Nrf2* gene promoter activity was measured by the luciferase activity assay (19). RPTCs stably transfected with the plasmid pGL4.20 served as controls.

In additional experiments, stable transformant RPTCs were transiently transfected with *Nrf2* siRNA or scrambled siRNA, and the

effects of HG on *Nrf2* and *Agt* mRNA expression and their respective gene promoter activities were analyzed after 24 h of incubation.

Statistical Analysis

The data are expressed as means ± SEM. Statistical comparisons were made by Student's t-test or 1-way analysis of variance and the Bonferroni test as appropriate. $p<0.05$ values were considered to be statistically significant.

RESULTS

Pathophysiological Measurements in Mice

Table 2 reports physiological measurements in non-Akita WT mice, Cat-Tg mice, Akita mice and Akita Cat-Tg mice at week 16. Briefly, Cat overexpression had no effect on blood glucose, whereas it completely normalized SBP in Akita Cat-Tg compared to Akita mice. Cat overexpression markedly attenuated, but did not completely normalize the GFR, urinary albumin-creatinine ratio (ACR), kidney weight/tibia length and heart weight/tibia length ratios, urinary Agt and Ang II levels in Akita Cat-Tg compared to Akita mice. In contrast, Cat overexpression did not affect any of these parameters except ACR and RPTC volume in Cat-Tg compared to WT controls.

Histology

Consistent with earlier observations (8,13,19), Akita mice developed renal structural damage (**Supplemental Figure 1A**). The histologic changes include proximal tubule cell atrophy, tubular luminal dilatation with accumulation of cell debris, and increased extracellular matrix proteins in glomeruli and tubules. Cat overexpression markedly reversed but did not completely resolve these abnormalities in Akita Cat-Tg mice.

Cat immunostaining (**Figure 1A**) and Cat activity (**Figure 1B**) but not*Cat* mRNA expression (**Figure 1C**) were significantly lower in RPTs from Akita mice compared to non-Akita WT, Cat-Tg or Akita Cat-Tg mice. In contrast, Akita mice exhibited significantly higher ROS levels, quantified by lucigenin assay (**Figure 1D**), NADPH oxidase activity (**Figures 1E**) and *Nox4* mRNA, quantified by RT-qPCR (**Figures 1F**) than non-Akita WT and Cat-Tg mice, indicating presence of markedly higher levels of oxidative stress. These changes were normalized in Akita Cat-Tg.

Effect of Cat Overexpression on Agt, Nrf2, HO-1 and Keap1 Expression in Akita Mice

Immunostaining revealed significantly higher Agt (**Figure 2A**), Nrf2 (**Figure 2B**) and HO-1 expression (**Figure 2C**) in RPTCs from Akita mice

compared to non-Akita WT or Cat-Tg mice. Cat overexpression normalized Agt, Nrf2 and HO-1 expression in Akita Cat-Tg mice. In contrast, no significant differences in Keap1 expression were detected in RPTCs among the different groups (**Figure 2D**). WB for Agt, Nrf2, Keap1 and HO-1 (**Figure 2E, a and b**) and RT-qPCR for *Agt*, *Nrf2*,*Keap1*and *HO-1*mRNA expression (**Figure 2F, a-d**, respectively) from isolated RPTs confirmed these findings. Furthermore, increases in nuclear Nrf2 but not cytoplasmic Nrf2 levels were observed in RPTs in Akita mice as compared to WT and Cat-Tg mice. Cat overexpression attenuated the nuclear but not cytoplasmic Nrf2 levels in Akita Cat-Tg mice (**Figure 2E, c**).

Effect of HG and H_2O_2on Agt, Nrf2 and HO-1 Gene Expression in rRPTCs in Vitro

Consistent with our previous observations on HGregulation of *Agt* gene expression (**Supplemental Figure 1B-E**), HG stimulated *Nrf2* mRNA in RPTCs in a concentration- and time-dependent manner (**Figure 3A and B**, respectively). The HG stimulation of *Nrf2* mRNA expression was inhibited by apocynin, diphenyleneiodonium chloride (DPI), rotenone and Cat (**Figure 3C**). Likewise, SB 203580, but not PD98059 or wortmannin, prevented HG-stimulated increases in *Nrf2* mRNAexpression (**Figure 3D**). Furthermore, both PDTC and BAY-11-7082 inhibited HG-stimulated increases in *Nrf2* and *Agt* both at themRNA (**Figure 3E and F**,

respectively) and protein levels (**Figure 3G and H**, respectively). Similarly, both BAY-11-7082 and PDTC inhibited HG-stimulation of HO-1 expression at both the mRNA and protein levels (**Figure 3I and J,** respectively).

We used H_2O_2 to determine whether ROS could directly stimulate *Nrf2*, *Agt* and *HO-1* gene expression in RPTCs.Indeed, H_2O_2 stimulated *Nrf2*, *Agt* and *HO-1* mRNA expression in RPTCs and these effects were inhibited in the presence SB203580 and BAY-11-7082, but not wortmannin or PD98059 (**Figure 4A, 4B and 4C**, respectively). Consistently, H_2O_2 stimulated *Nrf2* and *Agt* gene promoter activity in RPTCs and these were inhibited by SB203580 and BAY-11-7082, but not wortmannin and PD98059 (**Figure 4D and 4E**, respectively). Most interestingly, transfection of pcDNA3.1 plasmid containing Flag-(*Rel A*)*p65* cDNA stimulated Nrf2 and Agt gene promoter activity in a dose-dependent manner (**Figure 4F and 4G**, respectively).

Effect of Nrf2 Activation on Nrf2 and Agt Gene Expression in rRPTCs in Vitro

To study the impact of Nrf2 on *Nrf2* and *Agt* gene expression in RPTCs, we used the Nrf2 activator oltipraz and inhibitor alkaloid trigonelline as well as*Nrf2* cDNA transfection. Oltipraz stimulated *Nrf2* and *Agt* mRNA expression in a concentration-dependent manner (**Figure 5A and 5B**, respectively). Trigonelline inhibited HGstimulation of *Nrf2* and

Agt mRNA expression in a concentration-dependent manner (**Figure 5C and 5D**, respectively). Transient transfection of *Nrf2* cDNA stimulated both *Nrf2* and *Agt* mRNA expression (**Figure 5E and 5F**, respectively) as well as their respective gene promoters (**Figure 5G and 5H**, respectively) . Of note, *Nrf2* mRNA levels in **Figure 5E** were quantified with primers specific to the 3'-untranslated region of rat *Nrf2* mRNA (**Table 1**). These data demonstrated that Nrf2 activation stimulates both *Nrf2* and *Agt* gene expression in RPTCs.

Next, we investigated the effects of Nrf2 knock-down on HGstimulation of *Nrf2* and *Agt* gene expression in RPTCs. Transfection of RPTCs with *Nrf2* siRNA reduced Nrf2 and Agt protein (**Figure 6A and 6B**, respectively) and mRNA (**Figure 6C and 6D,** respectively) expression in a concentration-dependent manner without affecting Keap1 mRNA expression (**Supplemental Figure 1F**) . Scrambled siRNA had no effect on Nrf2 and Agtexpression. *Nrf2* siRNA prevented HGstimulation of *Nrf2 and Agt* gene promoter activity in a concentration-dependent manner (**Figure 6E and 6F,** respectively**)**.

Identification of Nrf2-REs in rAgt Gene Promoter

Figure 7A represents a schematic diagram of the *rAgt* gene promoter with putative *Nrf2*-REs. Two putative *Nrf2*-REs, *agagccnn* and *tgagccnn*, were localized in nucleotides N-964 to N-959 and N-913 to N-908 upstream of the transcription starting site of the *rAgt* gene promoter and designated as distal *Nrf2*-RE (d*Nrf2*-RE) and proximal *Nrf2*-RE (p*Nrf2*-RE), respectively. Transient transfection of plasmid containing

nucleotides 1,495 and 1,031 upstream of the transcription starting site pGL4.20 (*Agt* N-1495/+18) and pGL4.20 (*Agt* N-1031/+18) displayed 15-fold higher promoter activity than the promoter-less plasmidpGL4.20 (**Figure 7B**). Further deletion to N-442 of the *rAgt* gene promoter pGL4.20 (*Agt* N-442/+18) significantly reduced promoter activity compared to pGL4.20 (*Agt* N-1495/+18) and pGL4.20 (*Agt* N-1031/+18), indicating that enhancer(s) might be localized within nucleotides N-1031 to N-443 of the r*Agt* gene promoter.

Oltipraz stimulated *Agt* gene promoter activity (pGL4.20 (*Agt* N-1495/+18)) (**Figure 7C**). Trigonelline inhibited oltipraz stimulation of *Agt* gene promoter activity in a concentration-dependent fashion (**Figure 7D**). Interestingly, deletion of either *dNrf2*-RE or p*Nrf2*-RE only partially attenuated the stimulatory effect of oltipraz, whereas deletion of both *Nrf2*-REs completely abolished it (**Figure 7E**). These data demonstrate thatoltipraz induction of *Agt* gene transcription requires both *Nrf2*-REs in the r*Agt* gene promoter.

Effect of Oltipraz and Trigonelline on Nrf2 and Agt Expression in Mice in Vivo

Immunostaining of RPTCs for Nrf2, Agt and HO-1 revealed significantly higher expression levels in WT mice treated with oltipraz. Trigonelline co-administration reduced Nrf2, Agt and HO-1 expression to levels similar that of non-treated mice (**Figure 8A, 8B and 8C,**

respectively). Trigonelline alone had no detectable effects on either gene expression. WB for Nrf2, Agt and HO-1 protein (**Figure 8D, 8E and 8F**, respectively) and RT-qPCR of *Nrf2*,*Agt*and *HO-1*mRNA expression (**Figure 8G, 8H and 8I**, respectively) confirmed these findings. Oltipraz also stimulated *Nrf2* mRNA expression in the mouse liver, which was prevented by trigonelline (**SupplementalFigure 1G**). Oltipraz, however, did not affect *Agt* mRNA expression in the liver (**SupplementalFigure 1H**). These data demonstrate that Nrf2 activation differentially stimulates renal *Agt* gene expression and RAS activation in mice *in vivo*. Furthermore, oltipraz enhanced ROS generation and this was prevented by trigonelline co-administration (**Supplemental Figure 1I**). However, no statistically significant differences in SBP were found between non-treated, oltipraz ± trigonelline treated mice (**Supplemental Figure 1J**).

DISCUSSION

ROS generation, deficient antioxidant defenses and dysregulation of RAS have long been implicated in the development of renal injury in diabetes. However, the underlying molecular mechanisms are far from being fully understood. Our present results document that selective Cat overexpression in RPTCs effectively suppresses Nrf2-stimulation of *Agt* gene expression, attenuates systemic hypertension and kidney injury in Akita Cat-Tg mice. Nrf2 activation by oltipraz stimulates both *Nrf2* and *Agt* gene expression in RPTCs, and reversal of these actions by trigonelline

or Nrf2 siRNA both *in vitro* and *in vivo*. These data indicate that Nrf2 activation by oxidative stress (secondary to hyperglycemia) stimulates intrarenal Agt gene expression and RAS activation, subsequently leading to hypertension and development of nephropathy and identify a novel mechanism underlying the protective role of Cat.

In the Akita mouse, an autosomal dominant model of spontaneous type 1 diabetes, the *Ins2* gene is mutated, closely resembling that in patients with type 1 diabetes (27, 28). In the present study, we detected marked increases in ROS generation, NADPH oxidase activity and *Nox4* mRNA expression in RPTCs of Akita mice compared to non-Akita mice. These changes were normalized in Akita Cat-Tg mice, indicating that oxidative stress is a major component of renal injury in Akita mice.

Our data indicate that mitigating oxidative stress via kidney-specific Cat overexpression protects Akita mice against development of hypertension. The mechanisms underlying elevated SBP in Akita mice are still largely unknown. Our present findings demonstrate significantly higher Agt expression in RPTCs as well as higher urinary Agt and Ang II levels in Akita than in non-Akita WT and Cat-Tg mice. Cat overexpression in RPTCs normalized these changes. These observations are consistent with the clinical findings of elevated intrarenal RAS gene expression in diabetic and hypertensive patients (29-33).

Consistent with reports that Cat activity and expression were down-regulated in diabetic rats (34, 35) and that hyperglycemia induced up-regulation of Nrf2 expression in endothelial cells (36), we observed that Cat activity and expression were down-regulated. While Akita mice

exhibited slightly decreased *Cat* mRNA expression, it did not differ significantly from that in WT mice. The reason for decreased Cat activity remains unclear. One possible explanation is that elevated ROS would result in decreased Cat activity without affecting the expression of Cat mRNA.

The precise mechanism(s) by which hyperglycemia leads to up-regulation of renal *Nrf2* and *Agt* gene expression in diabetes remains unclear. One possibility is that ROS or hyperglycemia enhances *Nrf2* activation via promoting its dissociation from Keap1 and translocation into the nucleus. Nrf2 would then bind to *Nrf2*-binding sites in the *Agt* gene promoter region and promote*Agt* gene expression. Indeed, our *in vitro* studies in rRPTCs confirmed that HG and oltipraz stimulate both *Nrf2* and *Agt* gene expression and these can be reversed by trigonelline, *Nrf2* siRNA, and pharmacological inhibitors of ROS, p38 MAPK and NF-κB. Intriguingly, transient transfection of *Nrf2* cDNA stimulated both *Nrf2* and *Agt* mRNA and their respective gene promoter activities in RPTCs. This effect could be explained by the presence of *Nrf2-RE*s in both *Nrf2* (37) and *Agt* gene promoters (38). Furthermore, Nrf2 exerts positive auto-feedback on *Nrf2* gene transcription (37). Consistently, deletion of d*Nrf2*-REs and p*Nrf2*-REs completely abolished the stimulatory effect of oltipraz on *Agt* gene transcription, demonstrating that Nrf2 stimulation of *Agt* gene expression occurs at the transcriptional level.

In WT mice, administration of oltipraz stimulated *Nrf2*,*Agt*and *HO-*

*1*gene expression in RPTCs, and these actions were reversed by trigonelline co-administration. In contrast, oltipraz stimulated *Nrf2* but not *Agt* gene expression in the liver. These findings highlight tissue-specific Nrf2 regulation of *Agt* gene expression.

The molecular mechanism(s) by which p38 MAPK and NF-κB signal *Nrf2* and *Agt* gene expression are not fully understood. A likely mechanism is that HG activates p38 MAPK signaling via ROS generation, as we reported previously (3, 4). Activated p38 MAPK would then phosphorylate Nrf2, resulting in dissociation from Keap1, as it has been reported for various cell lines (39-41). Another possibility is that p38 MAPK activates NF-κB and increases the dissociation of NF-κB subunit p65 from p50. The p65 subunit would then bind to Keap1 and release Nrf2 as reported in human embryonic kidney cells (HEK 293) (42). Alternatively, the activated p65 subunit likely translocates to the nucleus, binds to the NF-κB-RE(s) in the *Nrf2* and *Agt* gene promoter and subsequently enhances *Nrf2* and *Agt* gene transcription. Indeed, this possibility is supported by our observations that transfection of RelA/p65 subunit stimulates both *Nrf2* and *Agt* gene promoter activity in RPTCs. Furthermore, consensus NF-κB responsive DNA sequences: 5'-*GGG AAC TCC G*-3' and 5'-*GGG ATT TCC C*-3' have been identified in the nucleotides N-371 to N-362 and N-578 to N-569 of the rat Nrf2 (37) and rat Agt gene promoter (38), respectively. In contrast, Liu et al (43)reported that NFκB could directly repress Nrf2 signalling at the transcriptional level

by competing with Nrf2 for transcription co-activator CREB binding protein (CBP). We currently do not have an explanation for this discrepancy. Whether the Nrf2 released from the CBP (caused by competitive binding of p65 on CBP) as described by Liu et al. (43) could exert a "positive" feedback on Nrf2 gene transcription as suggested by Kwaket al. (37) is unknown. Thus, further studies are needed to define the precise relationship of NFκB signalling and Nrf2 gene transcription in RPTCs.

Studies in rodents with Nrf2 activators (bardoxolone methyl analogs RTA 405 and dh404) have yielded conflicting results. Bardoxolone methyl analogs were reported to have potent anti-diabetic effects in diet-induced diabetic mice and in rodent models of type 2 diabetes and obesity (44, 45). In sharp contrast, recent studies reported that bardoxolone methyl analogs increased albuminuria and blood pressure along with weight loss in Zucker diabetic fatty rats (46). In clinical trials, phase 2 studies with bardoxolone methyl analogs reported reductions in serum creatinine levels or increases in the estimated GFR in human subjects with type 2 diabetes with stage 3b or 4 chronic kidney disease (47), suggesting a renoprotective action. However, phase 3 clinical trials involving patients with stage 4 advanced diabetic kidney disease were discontinued in 2012 after 9 months of follow-up due to increases in mortality rate and heart failure and development of hypertension and albuminuria (48). Furthermore, the study that was discontinued did not show much slowing of change in GFR (48). Our present data demonstrate that Nrf2 activation

enhanced intrarenal Agt gene expression, suggesting that Nrf2 might exaggerate renal dysfunction via the activation of the intrarenal RAS.

In summary, our findings indicate ROS-evoked Nrf2-mediated *Agt* gene expression in diabetes models both *in vivo* and *in vitro,* and document that these changes can be prevented by selective overexpression of Cat in RPTCs. Our findings also imply an important role for oxidative stress-induced *Nrf2* in the development of hypertension and renal injury in diabetes by altering the activation of local intrarenal RAS.

ABBREVIATIONS

ACR, albumin-creatinine ratio; Agt, angiotensinogen; Ang II, angiotensin II; BW, body weight; Cat, catalase; DHE, dihydroethidium; DMEM, Dulbecco's Modified Eagle's Medium; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; GFR, glomerular filtration rate; H_2O_2, hydrogen peroxide; HA, human influenza virus hemagglutinin; HG, high glucose; HO-1, heme oxygenase-1; *Ins2*, insulin 2 gene; KAP, kidney-specific androgen-regulated protein promoter; Keap1, Kelch-like ECH-associated protein 1; MAPK, mitogen-activated protein kinase; NF-kB, nuclear factor kappa-light chain enhancer of activated B cells; *Nox4*, NADPH Oxidase 4; *Nrf2,* nuclear factor erythroid 2-related factor 2; PDTC, pyrrolidinedithiocarbamate ammonium; PI3-K, phosphatidylinositol 3-kinase; r, rat; RAS, renin-angiotensin system; REs, response elements; ROS, reactive oxygen species; RPTs, renal proximal tubules; RPTCs, renal proximal tubular cells; PCR, conventional polymerase chain reaction; RT-qPCR, real time-quantitative polymerase chain reaction; SBP, systolic blood pressure; siRNAs, small interfering RNAs; Tg, transgenic; WB, Western blotting; WT, wild type

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J.S.D.C. (Université de Montréal and CRCHUM – Tour Viger) contributed to the discussion, wrote, reviewed and edited the manuscript.

DISCLOSURE

None reported.

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FIGURE LEGENDS

Figure 1.Characterization of Akita Cat-Tg mice. (A) Immunohistochemical staining for Cat in male non-Akita WT, Cat-Tg, Akita and Akita Cat-Tg mouse kidneys with rabbit anti-bovine Cat polyclonal antibody. Magnification X200. Cat activity (B), Cat mRNA (C), ROS generation (D), NADPH oxidase activity (E) and *Nox4* mRNA (F) expression in RPTs of WT controls, Cat-Tg, Akita and Akita Cat-Tg mice. Values are expressed as means ± SEM, n=8 per group. **p<0.01; ***p<0.005; NS, not significant.

Figure 2. Agt, Nrf2 and Keap1 expression in Tg mouse kidneys at week 16. Immunohistochemical staining for Agt (A), Nrf2 (B), HO-1 (C) and Keap1 (D) in mouse kidneys. Magnification X200. (E) WB of Agt, Nrf2, Keap1 and HO-1 expression (a) and quantification of their expression (b) in RPTs from kidneys of WT controls, Cat-Tg, Akita and Akita Cat-Tg mice. The membranes were re-blotted for β-actin. Agt, Nrf2,Keap1 and HO-1 levels were normalized by corresponding β-actin levels. Values are expressed as means ± SEM (n=8). ***p<0.005; NS, not significant.(c) WB of Nrf2 in nuclear and cytoplasmic fraction of RPTs from kidneys of WT controls, Cat-Tg, Akita and Akita Cat-Tg mice. (F) RT-qPCR of *Agt* (a), *Nrf2* (b),*Keap1* (c) and HO-1 (d) mRNA expression in RPTs of WT controls, Cat-Tg, Akita and Akita Cat-Tg mice. Agt, Nrf2, Keap1 and HO-1 mRNA levels were normalized by corresponding β-actin

mRNA levels. mRNA levelsin non-Akita control littermates were considered as arbitrary unit 1. Values are reported as means ± SEM, n=8 (*p<0.05; **p<0.01; ***p<0.005; NS, not significant).WT (empty bars), Cat-Tg mice (light grey bars), Akita (solid black bars) and Akita Cat-Tg mice (dark grey bars).

Figure 3. Effect of HG on *Nrf2*, *Agt* and *HO-1* mRNA expression in rat RPTCs. Cells were incubated in various concentration of D-glucose for 24 h (A) or for various time periods (B) in the presence of one of the following antioxidants: apocynin (10^{-5} M), DPI (10^{-7} M), Cat (300 U) or Rotenone (10^{-6} M) (C) or MAPK inhibitors: wortmannin (10^{-6} M), PD98059 (10^{-5} M) or SB203580 (10^{-6} M) or (D) or NF-κB inhibitors: BAY-11-7082 (10^{-5} M) or PDTC (10^{-6} M)) (E and F). Cells were harvested and assayed for *Nrf2* or *Agt* mRNA by RT-qPCR. WB of Nrf2 (G) and Agt (H) expression in RPTCs cultured in HG medium in the absence or presence of inhibitors BAY-11-7082 (10^{-5} M) or PDTC (10^{-6} M). RT-qPCR of *HO-1* mRNA (I) and WB of HO-1 protein (J) expression in RPTCs cultured in HG medium in the absence or presence of the NF-kB inhibitors BAY-11-7082 (10^{-5} M) or PDTC (10^{-6} M). Cells incubated in medium containing 5 mmol/l D-glucose plus 20 mmol/l D-mannitol were considered as control (arbitrary unit 1). Results are reported as percentages of control values (mean ± SEM, n=3). *p≤0.05; **p≤0.01; ***p≤0.005; NS, not significant. Similar results were obtained in 2 separate experiments.

Figure 4. Effect of H_2O_2 on *Nrf2*, *Agt* and *HO-1* mRNA expression in rat RPTCs. RPTCs or cells stably transfected with the pGL4.20 containing *Nrf2* or *Agt* gene promoter were incubated with 10^{-6} M of H_2O_2 in 5 mM D-glucose for 24 h in the absence or presence of wortmannin (10^{-6} M), PD98059 (10^{-5} M), SB203580 (10^{-6} M), BAY-11-7082 (10^{-5} M) or PDTC (10^{-6} M)). Cells were harvested and assayed for *Nrf2* or *Agt* mRNA by RT-qPCR (A and B, respectively) or promoter activity of *Nrf2* (C) and *Agt* (D) gene by luciferase activity assay or assayed for *HO-1* mRNA (E) and HO-1 protein (F) by RT-qPCR and WB, respectively. Cells incubated in medium containing 5 mmol/l D-glucose were considered as control (arbitrary unit 1). Results are expressed as relative values to control (mean $\pm$ SEM, n=3). *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.005$; NS, not significant. Similar results were obtained in 2 separate experiments. Effect of *Flag-(Rel A)p65* cDNA transfection on *Nrf* (G) and *Agt* (H) gene promoter activity in RPTCs. *Nrf2* and *Agt* gene promoter activity was quantified by luciferase activity assay. Luciferase activity in cells transfected with the plasmid, pcDNA 3.1 was considered as control (100%). Results are reported as percentages of control values (means $\pm$ SEM, n=3). *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.005$; NS, not significant.

Figure 5.Effect of Nrf2 activator on *Nrf2* and *Agt* gene expression in rat RPTCs. Cells were incubated in various concentrations of the Nrf2 activator oltipraz for 24 h (A) and (B) in the absence or presence of

various concentrations of the Nrf2 inhibitor alkaloid trigonelline (C and D). Effect of *Nrf2* cDNA transfection on *Nrf* (E) and *Agt* mRNA (F) expression in RPTCs. Cells were harvested and assayed for *Nrf2* or *Agt* mRNA by RT-qPCR. Effect of *Nrf2* cDNA transfection on *Nrf* (G) and *Agt* (H) gene promoter activity in RPTCs. *Nrf2* and *Agt* gene promoter activity was quantified by luciferase activity assay. Cells incubated in a medium containing 5 mmol/l D-glucose were considered as control (100%). Results are reported as percentages of control values (means ± SEM, n=3). *p≤0.05; **p≤0.01; ***p≤0.005; NS, not significant. Similar results were obtained in 2 separate experiments.

Figure 6. Effect of *Nrf2* siRNA on *Nrf2* and *Agt* gene expression in RPTCs in HG medium. Dose-dependent effect of *Nrf2* siRNA or scrambled siRNA on *Nrf2* (A) and Agt (B) protein expression in RPTCs incubated in HG medium and quantified by WB. Dose-dependent effect of *Nrf2* siRNA or scrambled siRNA on *Nrf2* (C) and *Agt* (D) mRNA expression in RPTCs incubated in HG medium and quantified by RT-qPCR. Dose-dependent effect of *Nrf2* siRNA or scrambled siRNA on *Nrf2* (E) and *Agt* (F) gene promoter activity in RPTCs incubated in HG medium was quantified by luciferase activity assay. Cells were harvested after 24 h of incubation. *Agt* mRNA levels in cells incubated in normal glucose medium are expressed as arbitrary unit 1. The results are reported as

percentages of control values (means ± SEM, n=3). *p<0.05; **p<0.01; ***p≤0.005; NS, not significant. Sc, scrambled.

Figure 7.Idenfication of *Nrf2*-REs in the Agt gene promoter. (A) Schematic diagram of *rAgt* gene promoter with putative *Nrf2*-REs. (B) Activity of plasmid containing various lengths of *Agt* gene promoter assayed by luciferase assay. (C) Concentration-dependent effects of the Nrf2 activator oltipraz on Agt gene promoter activity in RPTCs in 5 mM D-glucose medium in the presence or absence of the Nrf2 inhibitor trigonelline (D). (E) Effect of oltipraz on full-length *Agt* gene promoter activity with or without *Nrf2*-RE (d*Nrf2*-RE or p*Nrf2*-RE or both). The cells were incubated in medium containing 5 mM D-glucose for 24 h and then harvested. The results are reported as percentages of control values (means ± SEM), n=3 independent experiments (*p<0.05; **p<0.01; ***p<0.005; NS, not significant).

Figure 8. Effect of oltipraz on *Nrf2*, *Agt* and *HO-1* mRNA expression in mice *in vivo*. Immunohistochemical staining for Nrf2 (**A**), Agt (**B**) and HO-1 (**C**) in the kidneys of WT mice ± oltipraz and trigonelline. Magnification X200. WB of Nrf2 (**D**), Agt (**E**) and HO-1 (**F**) expression, and RT-qPCR of *Nrf2* (**G**), *Agt* (**H**) and *HO-1* (**I**) mRNA expression in RPTs of WT mice ± oltipraz and trigonelline. Values are expressed as

means ± SEM (n=6 per group). *$p<0.05$; **$p<0.01$; ***$p<0.005$; NS, not significant.

Table 1: Primer sequences for RT-qPCR, site-directed mutagenesis and sub-cloning

Gene	Species	Forward/reverse primer sequences	Accession number
Angiotensinogen	Rat / Mouse	F: 5'-CCACGCTCTCTGGATTTATC-3' R: 5'-ACAGACACCGAGATGCTGTT-3'	NM_007428.3
KEAP1	Rat / Mouse	F: 5'-CATCCACCCTAAGGTCATGGA-3' R: 5'-GACAGGTTGAAGAACTCCTCC-3'	NM_016679.4
Catalase	Rat / Mouse	F: 5'-CGACCAGATGAAGCAGTGGA-3' R: 5'-CCACTCTCTCAGGAATCCGC-3'	NM_009804.2
NOX4	Rat / Mouse	F: 5'-TGGCCAACGAAGGGGTTAAA-3' R: 5'-GATGAGGCTGCAGTTGAGGT-3'	NM_015760.4
Nrf2	Rat / Mouse	F: 5'-CGCCGCCTCACCTCTGCTGCCAGTAG-3' R: 5'-AGCTCATAATCCTTCTGTCG-3'	NM_010902.3
HO-1	Rat / Mouse	F: 5'-CACCAAGTTCAAACAGCTCT-3' R: 5'-CAGGAAACTGAGTGTGAGGA-3'	NM_010442.2
Nrf2 3'-UTR	Rat	F: 5'-GAAATGCAGAAACACTTTATAAG -3' R: 5'-GACTGTAACAAATGAGAACAG -3'	NM_031789.2
Nrf2 (cDNA)	Human	F: 5'-AAAGGTACCATGATGGACTTGGAGCTG CCG-3' R:5'-AAACTCGAGCTAGTTTTTCTTAACATCTGGC-3'	NM_006164.4
Nrf2 Promoter	Rat	F: 5'-GAACCATGATGATAATTAAGTCTCAG-3' R: 5'AAACTCGAGCTGGGACTGTAGTCCTGGCGG-3'	NM_003807581.1
Agt promoter (442bp)	Rat	F: 5'-AAAGGTACCGGTGCGGGAAGGGACTGG -3' R: 5'-AAAAAGCTTCCAGACAAGCACAGCTAT -3'	NW_003812957.1
Agt promoter (1044 bp)	Rat	F:5'-AAAGGTACCCCTCCAACAACTGGCTTCC-3' R: 5'-AAAAAGCTTCCAGACAAGCACAGCTAT -3'	NW_003812957.1
Agt promoter - ΔNrf2 prox.	Rat	F: 5'-CTAGTTTCTTCAGGGACTGCTCTGCC 3' R: 5'-GGCAGAGCAGTCCCTGAAGAAACTAG-3'	
Agt promoter - ΔNrf2 distal.	Rat	F:5'-GAAGGTCACTCTCTCAGCTCAGACACCATC -3' R:5'-GATGGTGTCTGAGCTGAGAGAGTGACCTTC -3'	
β-Actin	Rat, Mouse & Human	F: 5'-ACGATTTCCCTCTCAGCTT-3' R: 5'-TACAATGAGCTGCGTGTGGC-3'	NM_031144.3

Table 2. Physiological Measurements

	WT	CAT-Tg	Akita	Akita CAT-Tg
Blood glucose(mmol/L)	9.62±0.75	10.01±0.68	33.49±0.66***	33.80±0.41***
Systolic blood pressure (mmHg)	103.88±3.50	101±4.25	137.34±2.57***	117±3.75***†††
Body weight (g)	32.36±0.80	32.71±0.39	23.82±0.49***	24.84±0.53***
Kidney weight (mg)	370±10	380±10	540±24***	490±10***,†
Heart weight (mg)	140±10	140±10	160±10	150±10
Tibia length (mm)	18.5±0.12	18.4±0.14	16.5±0.10***	17.4±0.11**,††
Kidney/Tibia length (mg/mm)	20± 1.11	20.65±1.43	32.74±1.36***	28.10±0.32***,††
Heart/Tibia length (mg/mm)	7.56±0.10	07.60±0.10	9.63±0.31***	8.64±0.20*,†
Albumin/Creatinine ratio (µg/ml/mg/dL)	0.23 ±0.03	0.17 ±0.03	3.85 ±0.81***	2.2 ±0.66***††
GFR (ml/min)/BW (g)	9.92±0.80	10.64±0.77	22.75±2.15***	16.14±1.55**††
Glomerular tuft volume ($X10^3 µm^3$)	127.45 ±4.05	131.06 ±3.11	231.39±17.21***	171.33±6.51**,†††
RPTC volume ($X10^3 µm^3$)	5.44 ±0.11	4.82 ±0.05*	9.68±0.26***	5.86 0.14†††
Tubular luminal area ($µm^2$)	51.38±5.37	48.76±5.54	105.75±14.55**	66.27±9.11*,††
Urinary Agt/ Creatinine Ratio (ng/mg)	28.14±4.73	28.60±5.91	394.75±91.72***	220.81±23.48***,†
UrinaryAngII/ Creatinine Ratio (ng/mg)	1.20±0.42	3.56±1.34	38.64±12.04**	12.10±5.01**,†

***p<0.05; **p<0.01; ***p<0.005 vs WT; †p<0.05; ††p<0.01; †††p<0.005 vs Akita**

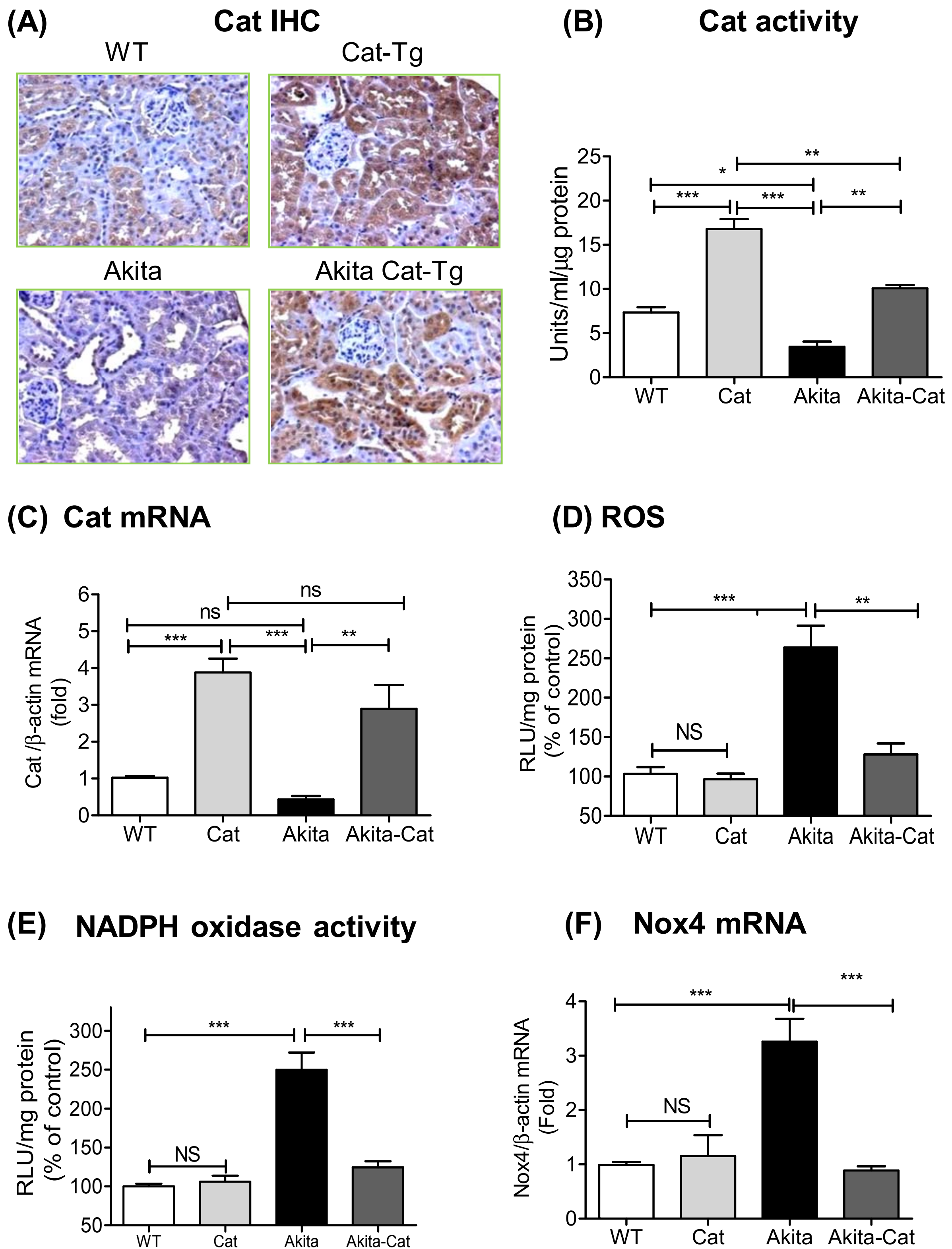


Figure 1

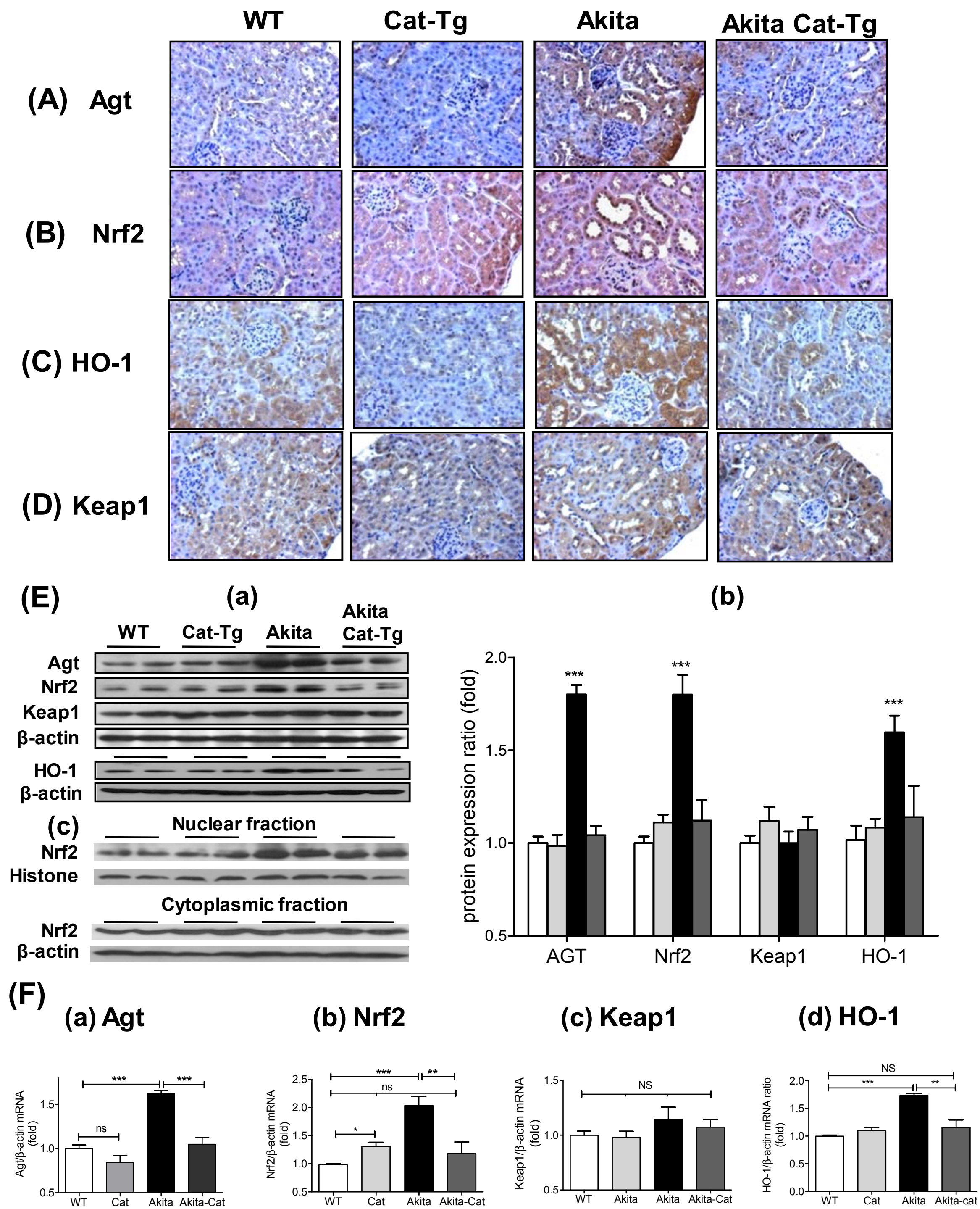


Figure 2

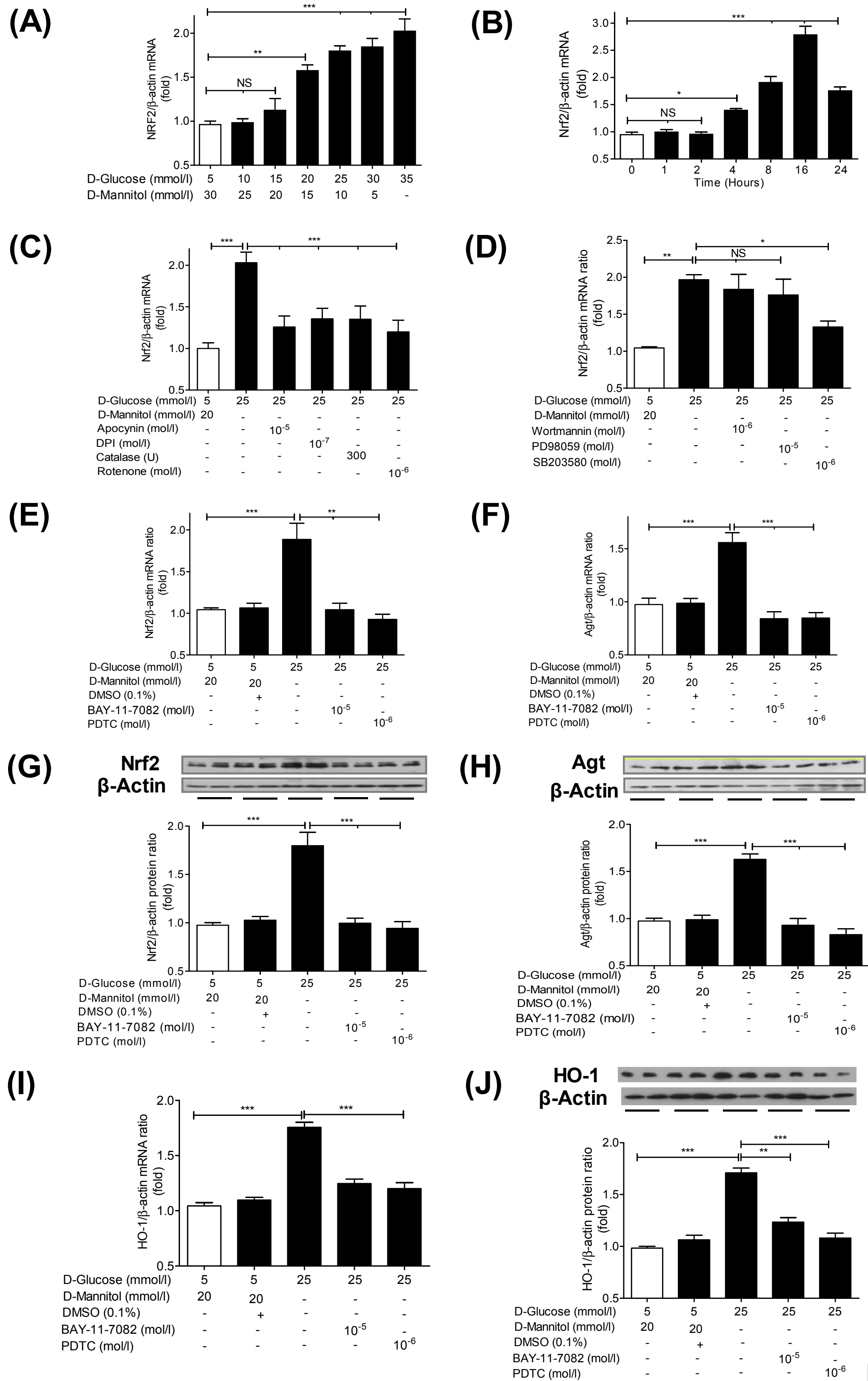
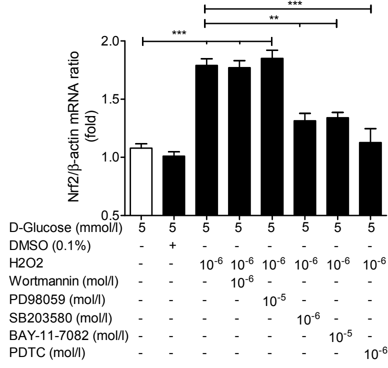
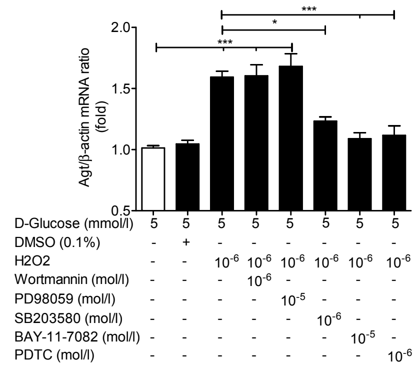
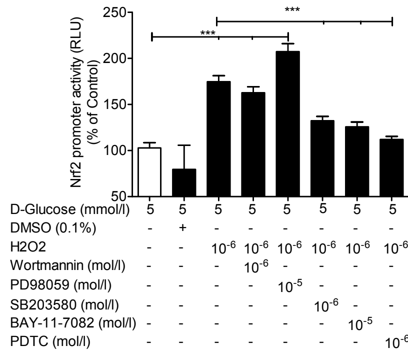
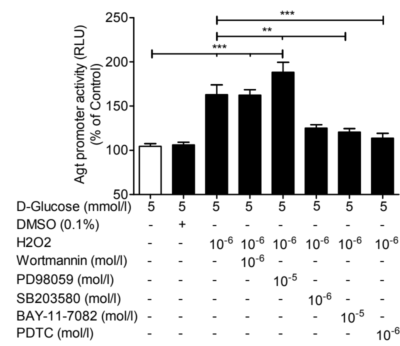


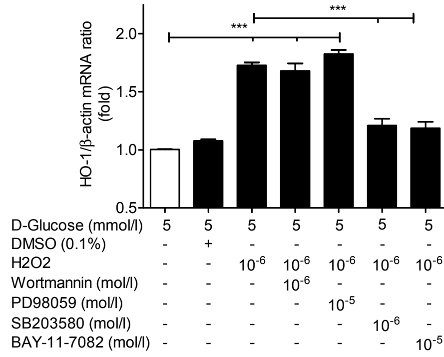
Figure 3

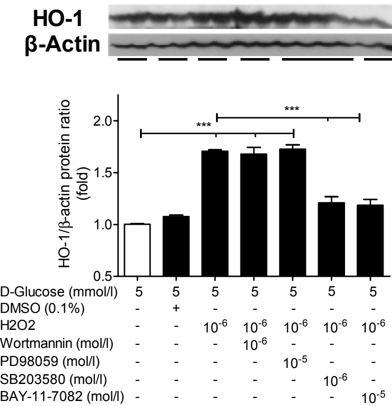


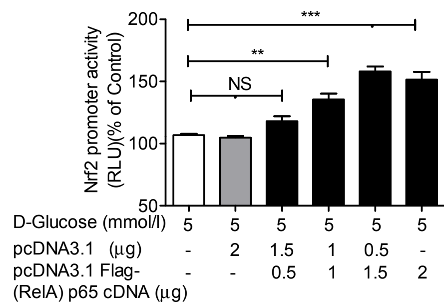












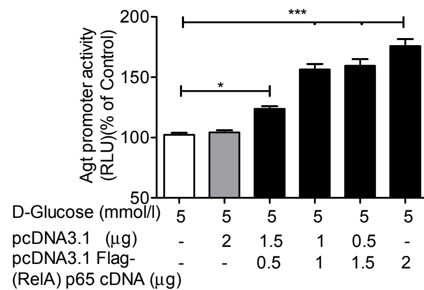


Figure 4

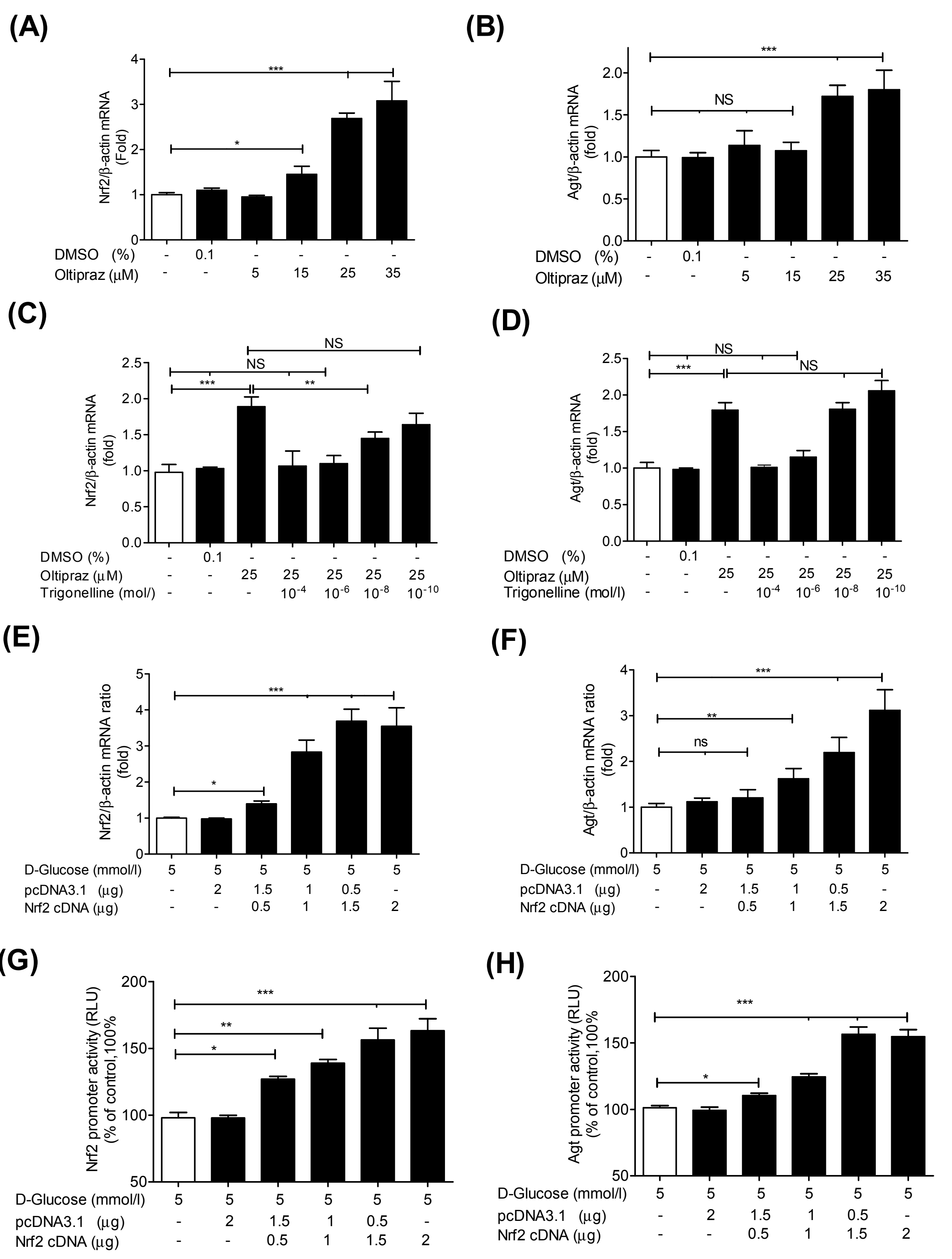


Figure 5

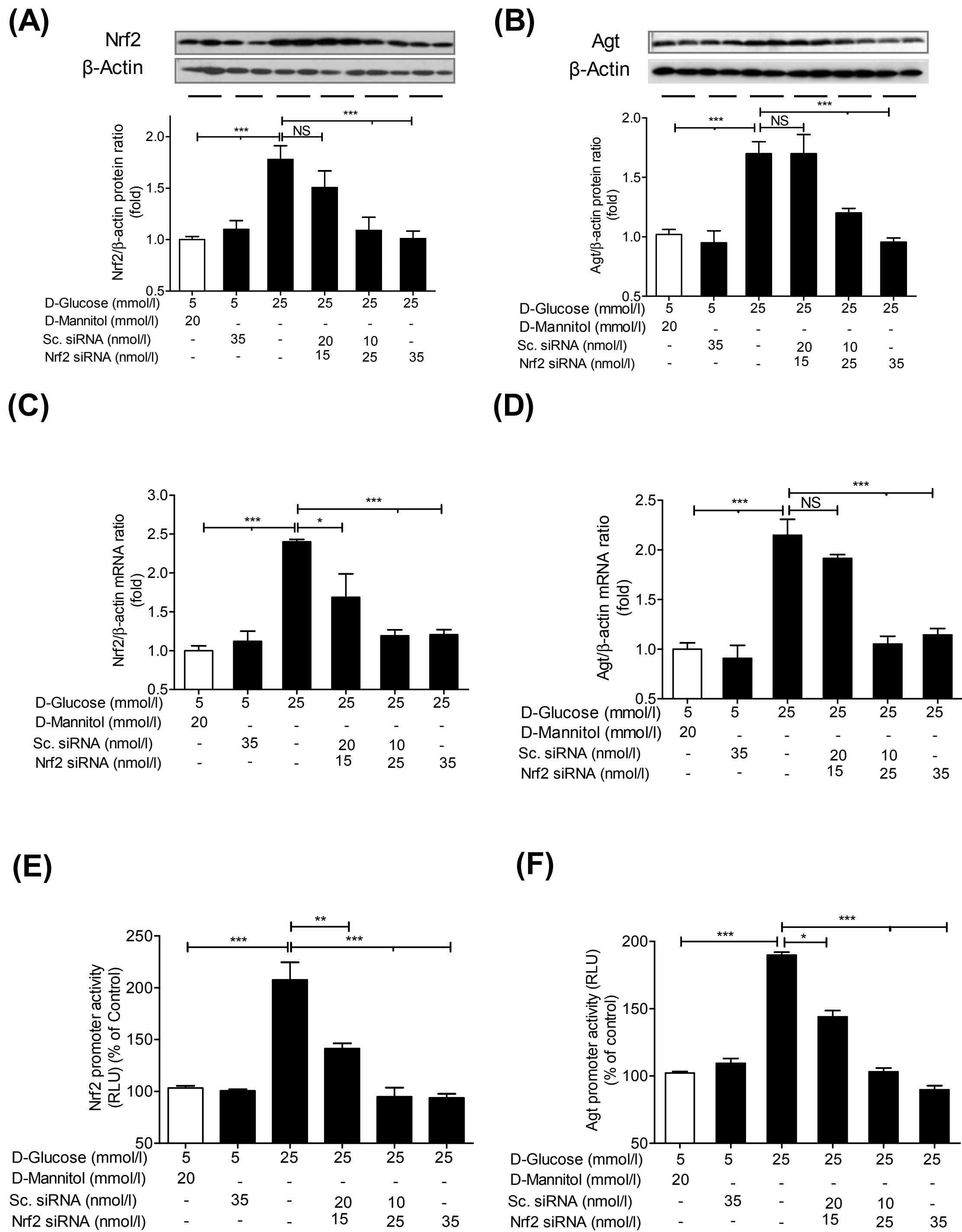


Figure 6

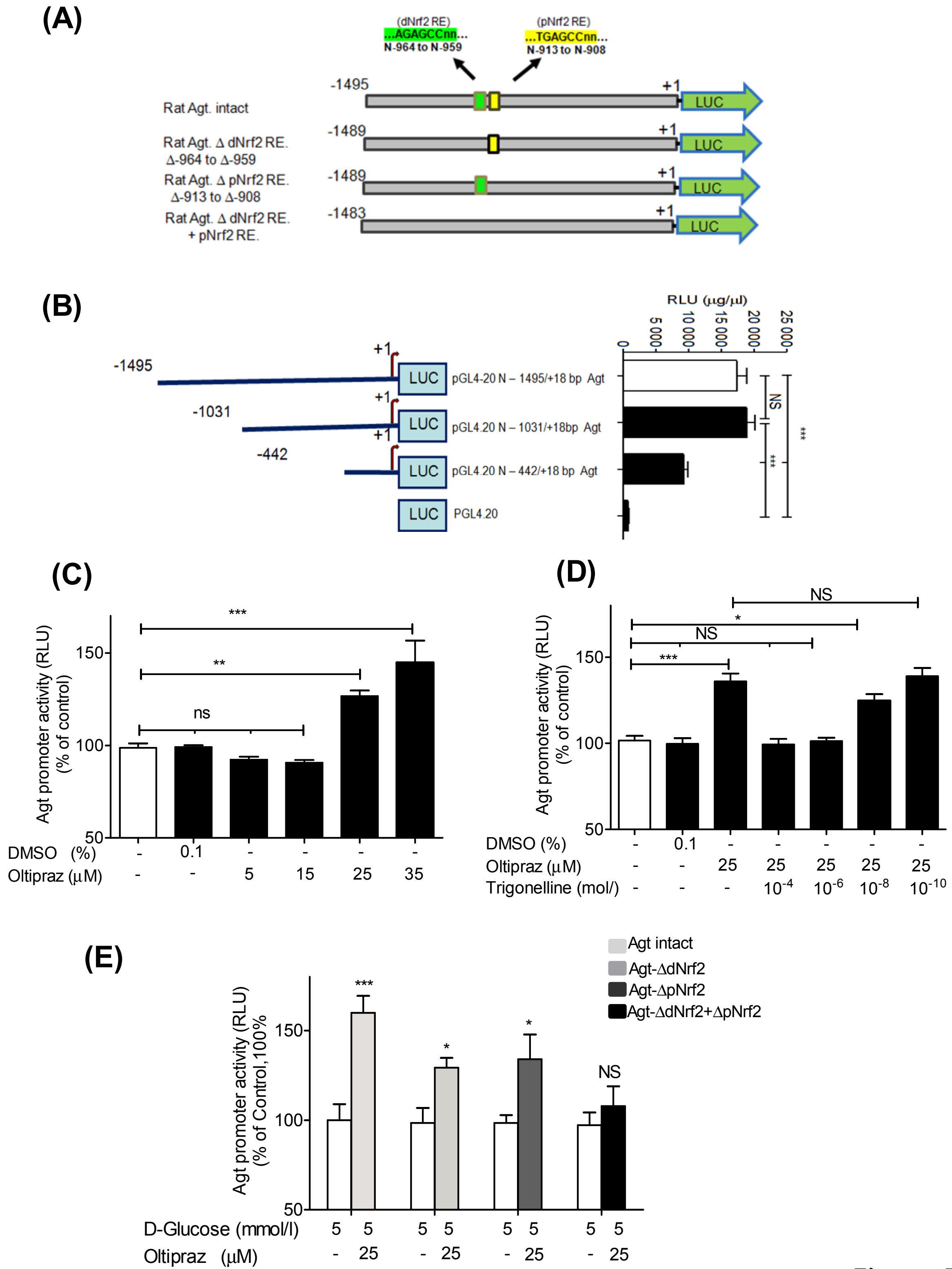


Figure 7

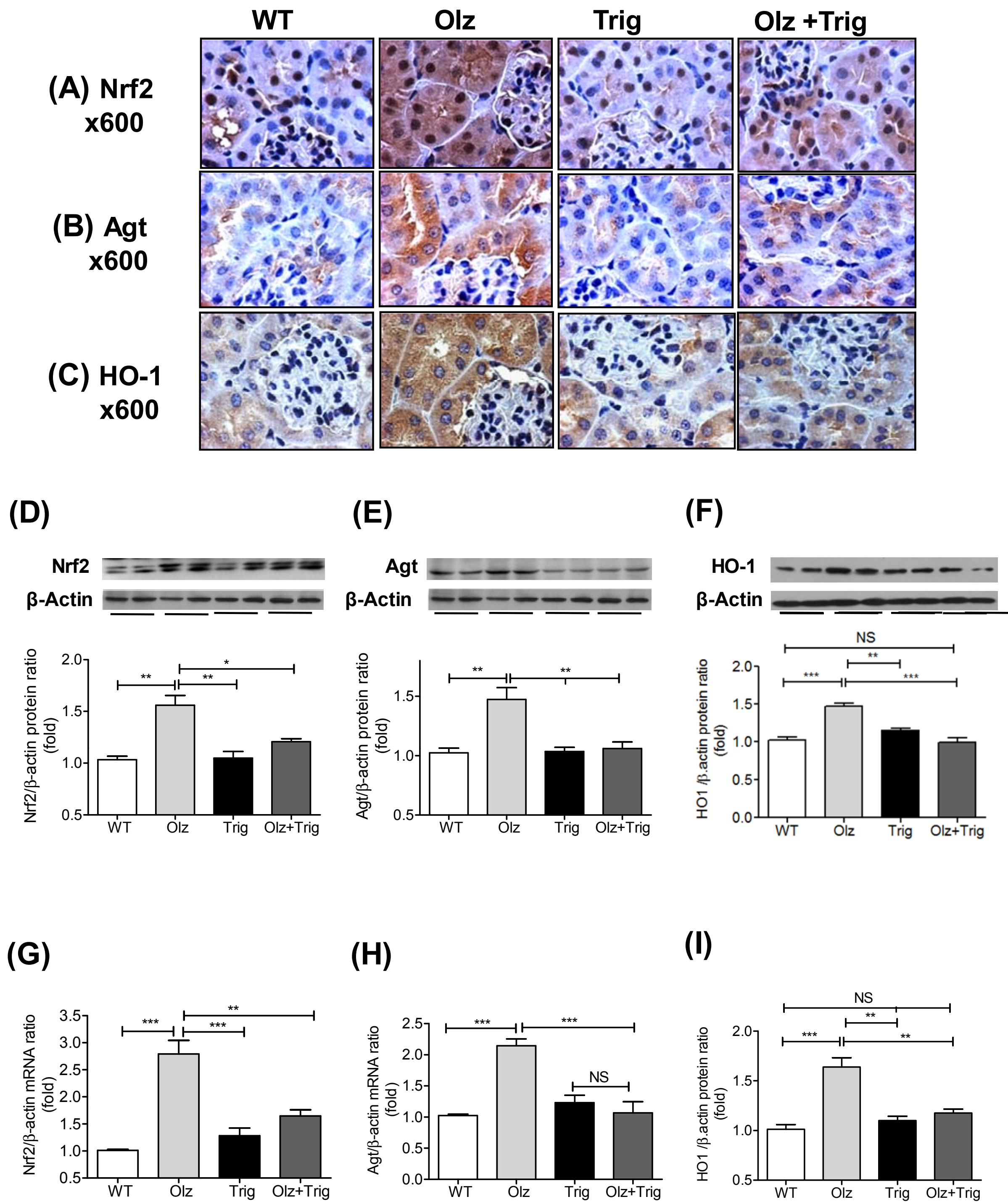
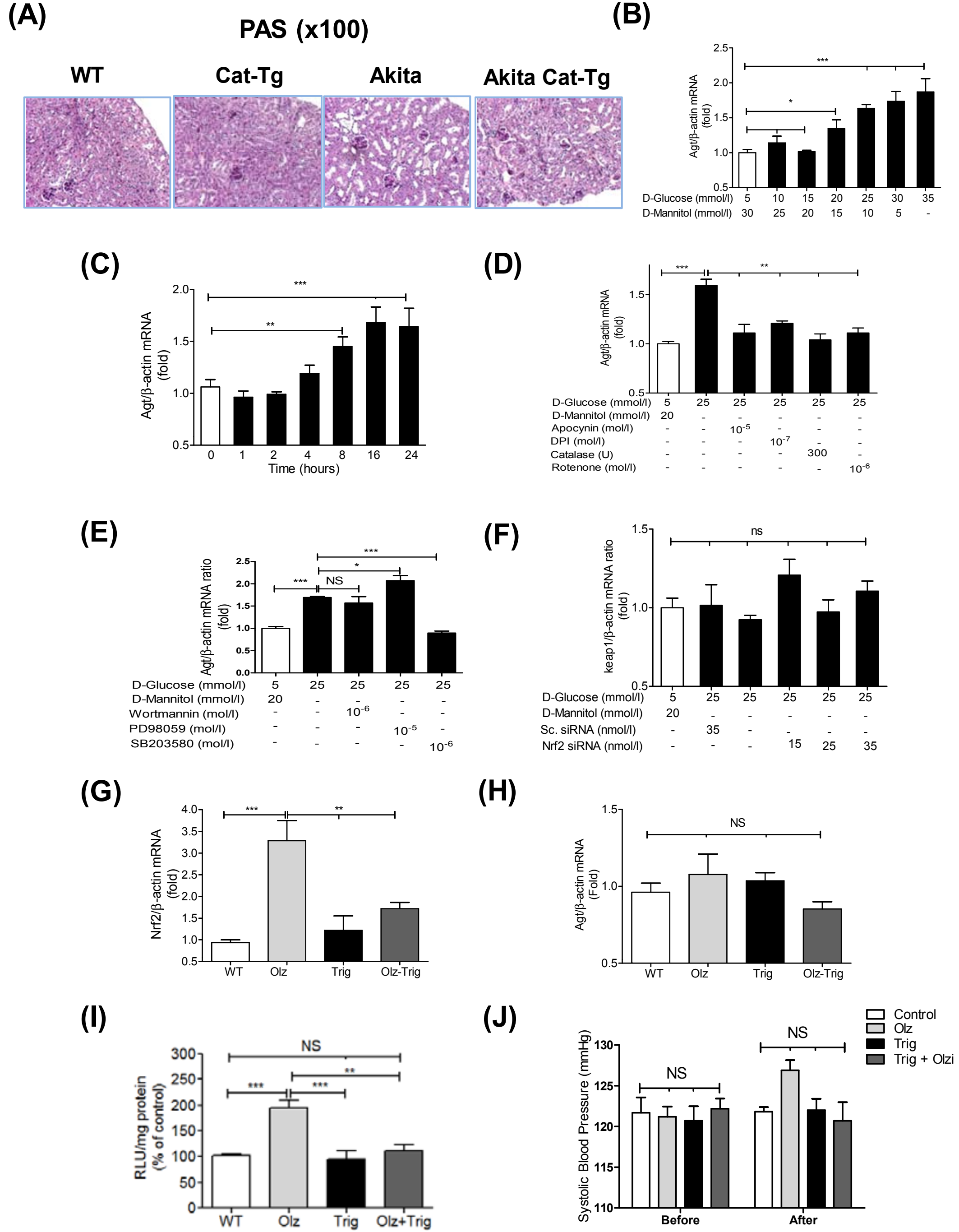


Figure 8

Supplemental Figure 1



Chapter 4 : Discussion

Hyperglycemia, oxidative stress and renin-angiotensin system Hyperglycemia, oxidative stress and renin-angiotensin system dysfunction have been implicated in the progression of DN, however, the underlying molecular mechanisms are far from being fully understood. DN is characterized by multiple structural and functional abnormalities, specifically at the level of the glomerulus, tubulointerstitium and vasculature of the kidneys of diabetic patients. Early tubular injury has been reported in DN patients whose glomerular function is intact [420]. The activation of the RAS and uncontrolled blood glucose are two of the major risk factors for the development of DN. Hyperglycemia in Akita mice begins at approximately 3-4 weeks of age. Our findings in chapter 2 demonstrate the effect of insulin treatment in Akita mice (for 4 weeks) on physiological and histological levels of the kidney. Blood glucose level, hypertension, ACR, GFR, urinary levels of ANG II and Agt were significantly elevated in Akita mice compared with WT controls. Insulin treatment decreased, though never completely normalized, the hyperglycemia level, SBP, kidney functions and urinary ANG II and Agt levels in Akita mice.

Histological findings included collagenous components and tubular luminal dilation with an accumulation of cellular debris in the tubular lumen; these findings were increased in Akita mice compared to WT. Insulin treatment of Akita mice markedly reversed, though never completely resolved these abnormalities. These data indicate the effect of insulin treatment on preventing tubulointerstitial fibrosis in Akita mice. The *Agt* gene expression was elevated in the RPTCs of adult Akita mice, although the underlying mechanisms remain incompletely understood. In contrast, the RPTCs of Akita mice exhibited decreased *hnRNP F* and *K* as well as *ACE-2* gene expression compared to WT controls. Insulin treatment increased the expression of *hnRNP F*and *hnRNP K*, normalized *ACE-2* gene expression and downregulated *Agt* expression in Akita mice.

To elucidate the possible mechanism of action underlying insulin activation of *hnRNP F* / *hnRNP K* and *Agt* downregulation we used immortalized rat RPTCs. The cells were obtained from Dr. Julie R. Ingelfinger of Harvard Medical School of Boston [421]. Immortalized rat RPTCs were derived from 4 to 6 weeks old WKY male rats with an origin-defective SV40 plasmid. The IRPTC line expresses all the components of RAS [422]. We examined immortalized rat RPTCs stably transfected with control PGL4.20 plasmid and PGL4.20 containing approximately1.5kb of rat *Agt* gene promoter, with and without insulin in high glucose milieu. We found that insulin inhibited *Agt* promoter activity though activation of *hnRNP F* and *hnRNP K* gene expression. Knockdown of *hnRNP F* or *hnRNP K* by small interfering RNAs attenuated insulin inhibition of Agt expression in RPTCs. Transfection efficiency of *hnRNP F* siRNA or *hnRNP K* siRNA showed that endogenous *hnRNP F* or *hnRNP K* protein expression decreased by more than 50%, respectively (chapter 2 ESM fig2). Transfection with either *hnRNP F* siRNA or *hnRNP K* siRNA partially attenuated insulin inhibition of *Agt* gene expression. This observation is in agreement with the study by Wei et al [230] in which they demonstrated that *hnRNP F* and *hnRNP K* proteins form a heterodimer and bind to the IRE region of the *Agt* promoter to regulate the *Agt gene* expression. Thus, silencing one of the two TFs will not completely abolish the inhibitory effect of insulin, whereas a combination of both siRNAs is more effective in attenuating the insulin inhibition of *Agt* gene expression. Recently, Lo et al [423] reported that overexpression of *hnRNP F* in RPTs of Akita mice attenuated the SBP, though not completely normalized.These observations raise the following question : whether the selective overexpression of *hnRNP F* and *hnRNP K* in RPTCs of a diabetic mouse would be more effective in controling Agt gene expression and SBP? Moreover, TFs regulate multiple genes indifferent pathways which by silence will disrupt the canonical pathway of other regulated genes. For example, under normal conditions *hnRNP F*

expression promotes alternative splicing of insulin receptor gene [283] that might influence the insulin pathway and affect *Agt* gene expression. Thus *hnRNP F* knockdown could affect its functionas an alternative splicer for other genes that might play an important role(s) in *Agt* gene expression. However, this hypothesis requires further investigation.

Pharmacological inhibitors show that the action of insulin on *A*gt, *hnRNP F* and *hnRNP K* transcription is mediated via the p44/42 MAPK signaling pathway, confirming our earlier findings [115]. Our data demonstrate that *hnRNP F* and *hnRNP K* mediate insulin inhibition of renal *Agt* expression, prevention of hypertension and amelioration of kidney function. The effect of high glucose and insulin on RAS and *hnRNP F* and *K* expression is shown in figure 4-1.

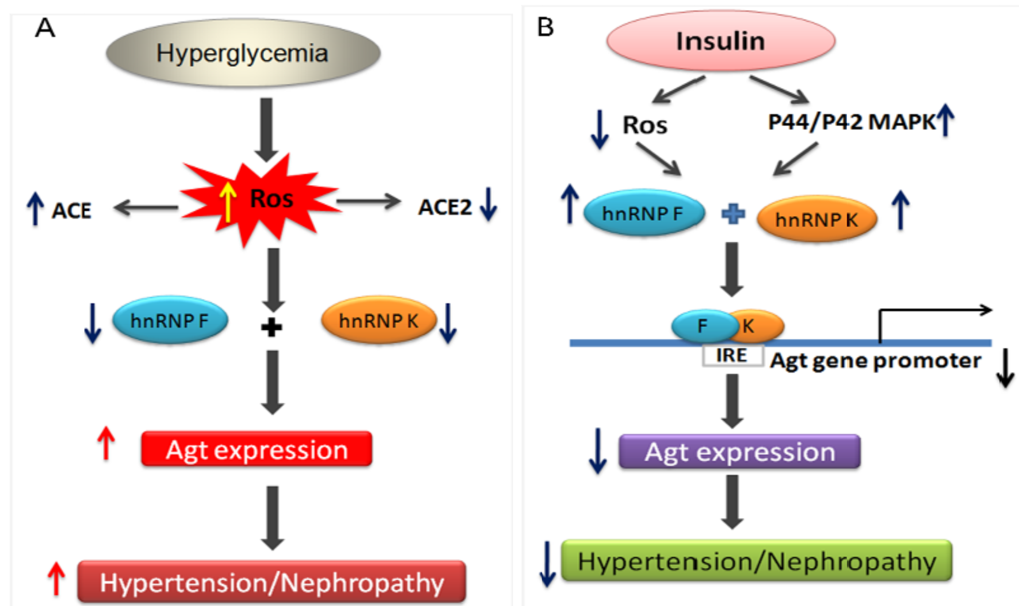


Figure 4-1 : The effects of high glucose and insulin on the RAS and hnRNP expression A: The effect of hyperglycemia on ROS activation, *Agt* gene expression and subsequent hypertension and nephropathy development. B: insulin effect of p44/p42 MAPK and downregulation of *A*gt gene expression via activation of hnRNPF and K.

4.1 Advantage of hyperinsulinemic-euglycemic clamp.

Insulin is a multifunctional hormone that plays a role in cell growth, glucose metabolism and could have a positive or negative effect on gene

transcription. Insulin regulates gene expression or induces post-translational modifications of preexisting molecules [424]. In chapter 2, we identify a novel mechanism underlying insulin inhibition of renal *Agt*expression and subsequent prevention of hypertension and kidney injury of Akita mice via the upregulation of renal *hnRNP F* and *hnRNP K*. Our experimental design has its limitations, namely that we cannot differentiate between a 'glucose-lowering effect' and a 'direct effect' of insulin on renal *Agt*, *hnRNP F* and *hnRNP K* gene expression in vivo. In order to elucidate the direct action of insulin on renal *Agt, hnRNP F* and *K* gene expression, we performed a hyperinsulinemic-euglycemic clamp in wild type mice in collaboration with Dr. Thierry Alquier of the CRCHUM. The hyperinsulinemic-euglycemic clamp is used to assess insulin action on gene expression and insulin sensitivity independent of its glucose-lowering effect in vivo [425]. This test is widely used as a standard reference to determine metabolic insulin sensitivity in humans [426]. Frequently, insulin infusion is accompanied by glucose infusion in order to maintain the basal glucose level and to prevent an insulin-induced drop in plasma glucose, meaning that the plasma glucose is kept at a normal range (euglycemic) during the period of the experiment [425]. The experiments have a duration of three hours, with elevation of insulin level up to 6 fold compared to control group (saline-infused group) as shown in figure 5-1.

In this experiment, we found that the gene expression levels of *hnRNP F* and *K* were increased in the insulin-infused group while *Agt* and *Nrf2* gene expression was decreased in mRNA and protein levels (unpublished results figure 5-2a-h). From these results it appears that insulin increases*hnRNP F* and *hnRNP K* gene expression and lowers the expression of *Agt* and *Nrf2* in renal proximal tubules compared to WT-saline group. Furthermore, IRPTCs that have been stably transfected with the pGL4.20 plasmid containing either the rat *hnRNP F* or the rat *hnRNP*

K gene promoter were also studied. High glucose decreased the promoter activity of *hnRNP F* and *hnRNP K*, while insulin treatment increased *hnRNP F* and *hnRNP K* gene promoter activity in both NG and HG milieu (figure 5-8). These results clearly demonstrate that insulin itself might play a role in the regulation of *hnRNP F, hnRNP K* and *Agt* gene expression. Furthermore, these results reinforce our earlier hypothesis that dysregulation of *hnRNP F* and *hnRNP K* expression in vivo may directly alter the activation of intrarenal RAS and, therefore, contribute to hypertension and renal injury in diabetes. Thus, hnRNP F and hnRNP K may be potential targets in the treatment of hypertension and kidney injury in diabetes.

Chan's group previously reported that *Agt* mRNA expression is upregulated in RPTs of STZ-induced diabetic mice. STZ drug is highly genotoxic; it causes DNA methylation, produces DNA strand breaks, chromosomal aberrations, micronuclei, and cellular death. STZ is also carcinogenic; a single administration induces tumors in the rat kidney, liver, and pancreas [329]. To avoid the 'nephrotoxicity' associated with STZ [331] and to demonstrate the physiological role of *hnRNP F*, *hnRNP K* and Nrf2, we used spontaneously diabetic Akita mice. The phenotype of the Akita mouse is similar to that of the human type 1 diabetic patient. Akita mice develop hyperglycemia, hypertension, and high oxidative stress leading to cardiovascular damage and glomerulosclerosis [427]. Agt is a glycoprotein that is the sole source of multiple angiotensins. Studies have demonstrated that *Agt* mRNA and protein are expressed in RPTs. In RPTs of Akita mice, we demonstrated that HG induces an increase in *Agt* gene expression at the transcriptional and translational levels, whereas insulin implant or Cat overexpression attenuated *Agt* gene expression as well as normalized systolic blood pressure (as described in detail in Chapter 2 and 3).

4.3 Promoter and transgenic mice.

Animal models are used to study the function of certain genes and their role in disease progression. Tg animal models help us understand the etiology of hypertension and develop strategies to control the disease progression. Organ-specific Tg mice is one of the recent advances in the Tg model in which expression of the transgene is tissue or organ-specific by organ-specific promoter control. For example, in the vascular system, Michael Bader generates Tg rats that overexpress human *ACE2* in the vascular smooth muscle under the control of the SM22 promoter [371]. In the heart, overexpression of human *AT1R* in mouse cardiomyocytes was derived by its expression under the control of the alpha-myosin heavy chain promoter [364], while the same gene was overexpressed in the rat podocyte under the control of a nephrin promoter [365]. In recent years, extensive attention has been given to local or tissue RASs. Among these, the intrarenal RAS expression is of special interest to us.

4.3.1 Androgen-regulated protein (KAP) promoter

The KAP is one of the most abundantly expressed genes in the proximal convoluted tubules [428]. The androgen-regulated protein (KAP) promoter has been used to drive the expression of transgenes in the RPTCs. This promoter is androgen-regulated and capable of specific targeting in RPTCs. In the human *Agt* Tg mouse, the construct consisted of 1542 base pairs of the KAP promoter fused with 10.3-kb of the human angiotensinogen (*hAgt*) gene (that included exons II, III, IV, and V, the intervening introns, a 70-bp segment derived from the 3'-end of intron I, and the native 3'-end of the *hAgt*gene containing the poly(A) sites). Studies have demonstrated the efficiency of the 1542 base pairs of the KAP promoter to drive the expression of the transgene into a tissue-specific, cell-specific, and androgen-regulated fashion in Tg mice. The KAP2 construct is a modified form of the KAP-hAgt construct characterized by the deletion of the coding sequence contained within the

hAgt exon II as well as creating a unique Not-1 restriction site downstream of the KAP promoter sequence to allow for cDNA insertion of any gene at the Not-1 site. By using the KAP2 vector, Chan's group generates rAgt-Tg mice, rCat-Tg, and rat *hnRNP F*-Tg mice. By studying the phenotype of Tg mice that have been crossbred with diabetic mice, we are now able to understand the function of these genes in the development of hypertension as well as diabetic nephropathy progression. Fang et al. demonstrated that overexpression of *rAgt* in RPTCs increases tubular apoptosis in STZ diabetic mice [16]. Cat overexpression attenuated *Agt* expression [417] and apoptosis in diabetic mice, while preventing hypertension and tubular apoptosis in Agt-Tg mice [219]. Recently, Lo et al.reported that overexpressing *hnRNP F* in RPTC of Akita mice efficiently suppresses *Agt* gene expression and attenuates systemic hypertension, kidney hypertrophy, and glomerulotubular fibrosis. These studies suggest a protective role of *hnRNP F* in preventing ANG II-induced hypertension and kidney injury [423].

HnRNP K is a transcription factor that plays a role in alternative splicing as well as regulating gene expression [429]. Xiao et al.demonstrated the role of hnRNP K as an antiapoptotic gene, independent of p53, in hepatocellular carcinoma via the maintenance of high levels of endogenous caspase inhibitors, and also identified *hnRNP K* as a possible therapeutic marker for cancer treatment [430]. In vitro studies show that overexpression of *hnRNP K* in IRPTCs inhibits angiotensinogen gene expression, and binds to the IRE of the *Agt* gene [230]. In addition, *hnRNP F* and *hnRNP K* are able to form heterodimers 230]. We have reported that the expression of both *hnRNP F* and *hnRNP K* are lower in the Akita mice (Figure 2-3e, f). Taken together, we postulate that overexpression of *hnRNPK* might play a protective role in hypertension. To test this we created Tg mice overexpressing rat *hnRNP K* in their RPTCs using the KAP2 vector. The transgene was tagged with

39bp of a myc tag at the 5' end of rat *hnRNP K*. The construct is shown in figure 5-3a (unpublished data).

The pKAP2-rhnRNPK construct was microinjected into one-cell fertilized mouse embryos (performed by Dr. Zhu of IRCM). Out of 75 offspring born, only 4 Tg founders containing the transgene were identified by southern blotting (Figure 5-3*B*). Three of these founders were successfully cross-bred with C57/B6 WT mice to establish the Tg lines. Studying the expression of the transgene in various tissues by polymerase chain reaction (RT-PCR) showed that rat-myc-hnRNP K is overexpressed only in the kidney of line 292. We have further characterized the line 292. Male Tg mice express the transgene in the kidney but not in other tissues, and exogenous testosterone further enhances renal transgene expression as shown in figure 5-3b and C.

Our preliminary data showed that rat *hnRNPK* is overexpressed in mouse RPTs and did not have a damaging effect on kidney histology as shown by PAS staining and Masson's trichrome staining (figure 5-5). Evidently, further studies are needed in order to understand the effects of *hnRNP K* oxerexpression in vivo on *Agt*, *hnRNP F* and *Nrf2* gene expression as well as the apoptotic effect on the Akita mice. For that purpose we need to crossbreed hnRNPK-Tg mice with a diabetic mice model such as db/db (type 2 diabetes) or Akita (type 1 diabetes). An alternative way to study the effect of *hnRNP K* overexpression in RPTs is to feed the trangenic mice a high salt diet or induce hypertension by Ang II administration for four weeks or more. A follow up of the mice is conducted by measuring their body weight and blood pressure, and comparing the data collected with those of the non-transgenic mice. More details about studies that could be done with the hnRNP K-Tg are summarized in perspective studies.

4.4 *HnRNP F* and *K* knockout mice

Overexpression of *hnRNP F* in RPTCs of Akita mice plays a protective role in kidney injury and efficiently suppresses *Agt* gene expression [423]. It is important to study *hnRNP F* and/or*hnRNP K* knockout mice to investigate the physiological role of these two TFs on *Agt* gene expression and kidney injuries. Targeted gene disruption has become a tool to study the physiological role of certain genes. Conventional knockout technology has certain limitations in which the gene of interest to knockout could be essential for development and survival, causing embryonic or postembryonic lethality phenotype [431]. For example, homozygous deletion of mouse *hnRNP K* results in embryo lethality prior to embryonic day 13.5, whereas nearly 50 % of heterozygous *hnRNP* $K^{+/-}$ mice survive with developmental defects. Surviving *hnRNP* $K^{+/-}$ mice are at high risk to develop tumors and some develop hematological neoplasms[432]. *HnRNP F* knockout mouse is not yet available. Thus, conditional gene knockout represents an extremely powerful approach to overcome the limitation of conventional knockout [433]. In our lab Dr. Lo is working to create a conditional *hnRNP F* knockout mouse in the RPTCs using the Cre-*loxP* system. The Cre-*loxP* site-specific recombination system can be used to knockout genes, and thus analyse their function, at a single developmental stage or in specific cell types or tissues. Dr Lo generates mice harboring a conditional *hnRNP F* allele by flanking *hnRNP F*exon 4 with two *loxP*-recombination sites to generate a ''floxed'' *hnRNP F* allele. To obtain *hnRNP F* knockout mice in the RPTs, *hnRNP* $F^{floxed/\ floxed}$ (floxed mice) will breed with Tg mice expressing Cre recombinase under the control KAP2 promoter (KAP2-Cre mice). KAP promoter is critical to determine the site of Cre expression. Cre enzyme, in turn, recombines the floxed gene and produces gene knockout. By this approach we will be able to investigate the physiological role of *hnRNP F* knockout in the RPTs as well as its role in regulating Agt gene expression.

4.4 Catalase gene overexpression

Akita mice exhibit decreased numbers of β-cells in the pancreatic islets, develop hyperglycemia [340], hypertension and increases in oxidative stress markers in RPTs that lead to kidney damage [167]. Renal injury in the Akita mice is characterized by tubular luminal dilation, glomerular hypertrophy and increased RPTC volume.We detected marked increases in ROS generation, NADPH oxidase activity and *Nox4* mRNA expression in RPTCs of Akita mice compared to non-Akita mice (Chapter 3). These changes were normalized in Akita Cat-Tg mice, indicating that oxidative stress is a major component of renal injury in Akita mice.

An unbalanced production of ROS plays a role in the pathogenesis of different diseases. Protection against oxidative damage is accomplished by a complex defense system composed of antioxidant molecules (such as *Nrf2*) and antioxidant enzymes (*SOD*, *GSH* and *Cat*) [418] that converts excessive reactive species to less reactive and less damaging forms. Catalase plays a major role in cellular antioxidant defense by decomposing hydrogen peroxide, thereby preventing the generation of hydroxyl radical. In Akita, we detected that the enzyme activity of catalase was decreased compared to the control group (chapter 3 fig 1c), in addition to renal structural damage, including tubular luminal dilation, glomerular hypertrophy, and increased RPTC volume [167]. These findings are in agreement with a study done by Kobayashi et al.[434], which showed that a deficiency of catalase enzyme activity (acatalasemia) promotes enhanced oxidant tissue injury, and interstitial fibrosis leading to renal function impairment. Moreover, mice lacking catalase develop normally but show differential sensitivity to oxidant tissue injury [435]. The role of catalase in defending cells and tissues against oxidative stress has been studied extensively. Transgenic mice overexpressing various antioxidant enzymes have been generated by

several laboratories. Transgenic mice overexpressing rat *Cat* in their heart are protected against cardiotoxicity following doxorubicin treatment in these animals [436]. Chan's group has demonstrated that overexpression of rat *Cat* in RPTs attenuated ROS generation, *Agt* and proapoptotic genes expression in the kidneys of STZ-induced diabetic mice [417]. We demonstrate in chapter 3 that overexpression of *Cat* in RPTs of Akita mice attenuates the ROS level which is in agreement with the study done by Shi et al. [218] compared to control mice. Additionally, *Cat* overexpression in RPTs of Akita mice prevents hypertension, progression of nephropathy, and highlights the importance of intrarenal oxidative stress and renal injury in diabetes. *Cat* overexpression in pancreatic beta cells is a partial protection against some beta cell toxins and is compatible with normal function [437].

4.5 NADPH oxidase and Nox4.

The NADPH oxidase is a family of seven members that play a major role to catalyze the production of superoxides and other ROS.These are NADPH oxidase 1 (*Nox1*), NADPH oxidase 2 (*Nox2*), NADPH oxidase 3 (*Nox3*), NADPH oxidase 4 (*Nox4*), NADPH oxidase 5 (*Nox5*), Dual oxidase 1 (*Duox1*), and Dual oxidase 2 (*Duox2*) [438]. *Nox1*, *Nox2* and *Nox4* have been shown to be expressed in the renal cortex but *Nox4* is the most common Nox isoform to be expressed in the kidney [439, 440]. *Nox4* contributes to basal ROS production through its constitutive activity and to increased ROS generation when stimulated by Ang II, glucose, and growth factors [441, 442]. In vitro study shows that *Nox4* activation produced $H_2O_2$and not superoxide [443]. Additionally, Nox4 activation regulates *Nrf2* and glutathione redox in cardiomyocytes [444]. Our data in chapter 3 fig 1d-f, show that Akita mice exhibit significantly higher ROS levels, NADPH oxidase activity and *Nox4* mRNA than non-Akita WT and Cat-Tg mice, indicating presence of markedly

higher levels of oxidative stress. These changes were normalized by overexpressing *Cat* in Akita mice. Although the endogenous *Cat* expression is downstream the *Nox4* mRNA expression, overexpression of rat *Cat* under KAP promoter in RPTs of Akita mice normalizes the ROS level by inhibitingthe *Nrf2* translocation to the nucleus (figure- 2E [c]), which could lead tonormalizing *Nox4* transcription**.** Pendyala et al. demonstrated by CHIP assay that *Nrf2* binds to AREs regions of the human *Nox4* gene promoter and regulates its activity. Moreover, hyperoxia stimulated *Nrf2* translocation to the nucleus and the knockdown of *Nrf2*gene expression by siRNA approach attenuated hyperoxia-induced *Nox4* expression [445]. This revealed the important role of *Nrf2* in regulating *Nox4* gene expression. After analysis of 3kb of mouse *Nox4* gene promoter, the putative *Nrf2* binding site was found in the proximal region between nucleotide number -588and -598 relative to the transcription starting site. Additionally, the endogenous*Cat* gene expression in the Akita mice group is regulated by the ROS activity and *Nrf2* activation.

4.6 Advantages and Disadvantages of Nrf2 Activation

The *Nrf2–Keap1* pathway is one of the major protective processes. Under physiological conditions, the *Nrf2* signaling pathway is negatively regulated by *Keap1* [301]. In response to oxidative stress, *Nrf2* is responsible for regulating a series of antioxidant and cellular protective genes (such as *HO-1*, *NADPH* and glutamatecysteine ligase) to neutralize the effects of ROS [296]. Although *Nrf2* is translocated to the nucleus under high glucose or H_2O_2 conditions to activate antioxidant genes, Han et al. demonstrated that primary cultures of RPTCs treated with H_2O_2 and high glucose show a decrease in the activity of both endogenous antioxidants, *Cat* and glutathione (*GSH*), in the rabbit. In addition, the effect of high glucose on the reduction of *Cat* was blocked by

Rotenone, or Apocynin treatment [446]. STZ-induced diabetes in the rat exhibited low catalase activity in the cortex and medulla of these rats [447]. We observed that *Cat* activity and expression were downregulated whereas *Nrf2* and *Agt* expression were upregulated in the RPTCs of Akita mice. The precise mechanism by which hyperglycemia leads to up-regulation of renal *Nrf2* and *Agt* gene expression in diabetes still remains unclear.

ROS activation or hyperglycemia enhances *Nrf2* translocation into the nucleus. *Nrf2* would then bind to *Nrf2*-binding sites in the *Agt* gene promoter region and promote*Agt* gene expression. Indeed, our *in vitro* studies in rRPTCs confirmed that HG, Oltipraz, and transient transfection of *Nrf2* cDNA stimulate *Nrf2* and *Agt* gene expression. This effect could be explained by the presence of *Nrf2-RE*s in both *Nrf2* [448] and *Agt* gene promoters[449]. Consistently, deletion of d*Nrf2*-REs and p*Nrf2*-REs completely abolished the stimulatory effect of Oltipraz on *Agt* gene transcription, demonstrating that *Nrf2* stimulation of *Agt* gene expression occurs at the transcriptional level. In WT mice, administration of Oltipraz stimulated both *Nrf2* and *Agt* gene expression in RPTCs, and these actions were reversed by trigonelline co-administration. In contrast, Oltipraz stimulated *Nrf2* but not *Agt* gene expression in the liver. These findings highlight the tissue-specific *Nrf2* regulation of *Agt* gene expression.

When cells are under oxidative, electrophilic stress, or when treated with chemopreventive compound, they often develop mechanisms to overcome cellular damage to increase the chance for survival. Several genes containing the anti-oxidant responsive element (ARE) in their promoters that mediate the effects of *Nrf2* have been identified, and some of them have shown promise for cancer prevention [450].

Chemopreventive agents such as oltipraz, butylatedhydroxyanisole, and bardoxolone methyl stimulate the Nrf2-ARE signaling pathway and are used as chemotherapeutics based on stimulation of the Nrf2-ARE signaling pathway [451]. In contrast, several reports indicate that the persistent accumulation of *Nrf2* in the nucleus is harmful. Under oxidative or electrophilic stress, Maher et al.reported that *Nrf2* regulates the expression of several multidrug resistance-associated proteins (MRPs), which could lead to chemotherapeutic drug resistance [419]. Furthermore, *Nrf2* has been shown to play dual roles in cancer cells [452]. In some contexts, *Nrf2* is considered as a promising therapeutic target for cancer treatment, but this has to be counterbalanced by the fact that *Nrf2* activation can also cause cellular chemoresistance [453]. Therefore, it has been suggested that the *Nrf2* pathway be inhibited during chemotherapy [235]. Lau et al.demonstrate that deregulation of autophagy causes the accumulation of p62 that directly interacts with *Keap1*, resulting in the inhibition of *Keap1*-mediated *Nrf2* ubiquitination [454]. The non-ubiquitinated *Nrf2* activates the *Nrf2* pathway in a non-canonical cysteine-independent mechanism

Pergola et al.reported that severe cases of CKD and type 2 diabetes treated in the short term with Bardoxolone methyl show improvement in the eGFR, renal function, and kidney injury [455]. In contrast, Dick de Zeeuw et al. studied the effect of bardoxolone methyl in 2185 patients with T2D and stage 4 CKD to determine whether Bardoxolone methyl, an activator of the *Nrf2* pathway, would lower ESRD risk in these patients. Unfortunately, the bardoxolone methyl-treated group of patients showed significant increases in the estimated GFR, blood pressure, and the urinary albumin-to-creatinine ratio with decreases in body weight compared to the placebo group, as well as developed a higher risk of cardiovascular events. The study has been terminated prematurely due to safety concerns and it was concluded that

bardoxolone methyl did not reduce the risk of ESRD or the rate of death [456]. This finding leads us to consider that treatment with bardoxolone methyl may have adverse side effects, or that the activation of the *Nrf2* pathway could play a role in the increased blood pressure. To further explore these ideas, we have investigated the relationship between renal *Agt* gene expression and *Nrf2* activation in the RPTs of wild type mice (chapter 3). We found that Oltipraz administration increases *Nrf2* and renal *Agt* gene expression. Moreover, gene promoter analysis predicted two binding sites for *Nrf2* in the rat *Agt* gene promoter and by deleting these sites, the *Agt* promoter activity was restored to normal levels in high glucose media. These findings indicate that *Nrf2* might have dual roles: stimulation of anti-oxidant genes and hypertensinogenic genes.

In conclusion, controlling the glycemia in Akita diabetic mice by insulin implant for 4 weeks normalized systolic blood pressure, proteinuria, glomerular hyperfiltration, and the intrarenal expression of angiotensinogen via upregulation of *hnRNP F* and *hnRNP K*. In vitro study in IRPTCs showed that insulin decreased the expression of *Agt* and increased levels of *hnRNP F* and *hnRNP K*, possibly through activation of MAP kinases. Moreover, *hnRNP F* and *hnRNP K* regulate *Agt* gene expression, and knockdown of *hnRNP F* or *hnRNP K* prevented the effect of insulin on *Agt* gene expression.

Additionally, we investigated the effect of hyperglycemia/ hypo-insulinism in the modulation of *Agt* gene expression and *Nrf2* with and without overexpression of catalase (antioxidant effect) cDNA in the renal proximal tubule of Akita mice. Overexpression of catalase normalized blood pressure, decreased histological kidney lesions, and decreased expression of *Nrf2*, heme-oxygenase, and renal*Agt* in Akita mice compared to normal mice. Oltipraz administration increases *Nrf2*, *HO-1,* and renal *Agt* gene expression; these effectsare blocked by trigonelline,

acting as *Nrf2* inhibitor. Moreover, deletion of the *Nrf2* response elements of the promoter *Agt* abolished the stimulatory effect of this activator of *Nrf2* on the expression of *Agt*, suggesting a direct transcriptional action. In summary, our findings indicate the ROS-triggered *Nrf2*-mediated *Agt* gene expression in diabetes models both *in vivo* and *in vitro,* and document that these changes can be prevented by selective overexpression of *Cat* in RPTCs. Our findings also imply an important role for oxidative stress-induced *Nrf2* in the development of hypertension and renal injury in diabetes by altering the activation of local intrarenal RAS.

Chapter 5: Perspectives of Research and Unpublished Results

5.1Hyperinsulinemic-euglycemic clamp and gene expression.

We performed this experiment to investigate the direct action of insulin on renal *Agt, hnRNP F* and *K* as well as *Nrf2* gene expression. The hyperinsulinemic-euglycemic clampwas performed in two groups of wild type mice. The control group (7mice) was injected with salin and the other group (7 mice) was injected with insulin. The experimental design and results obtained are presented in fig 5-1 and 5-2.

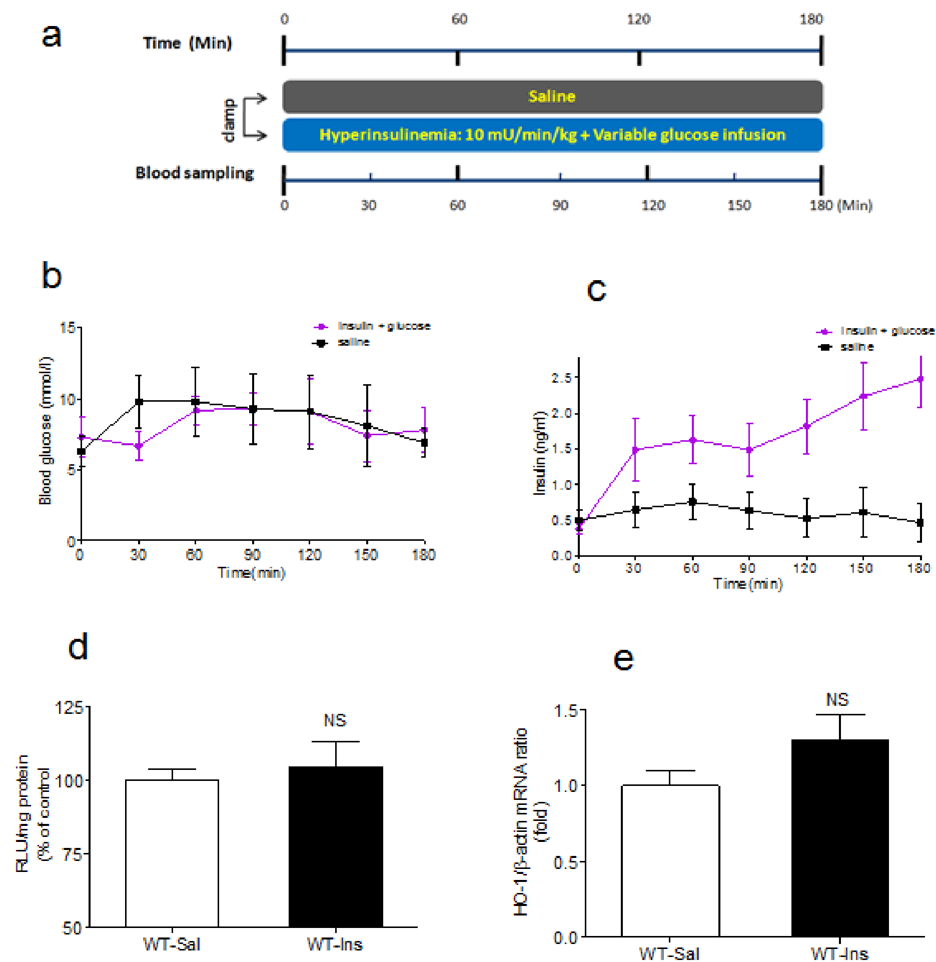


Figure 5-1; The Hyperinsulinemic-euglycemic Clamp (a), The experimental design for the animal groups and insulin infusion (b, c), blood glycemia and insulin levels in WT mice injected with saline (WT-sal) and insulin (WT-Ins). ROS level (d) and HO-1 mRNA gene expression (e).

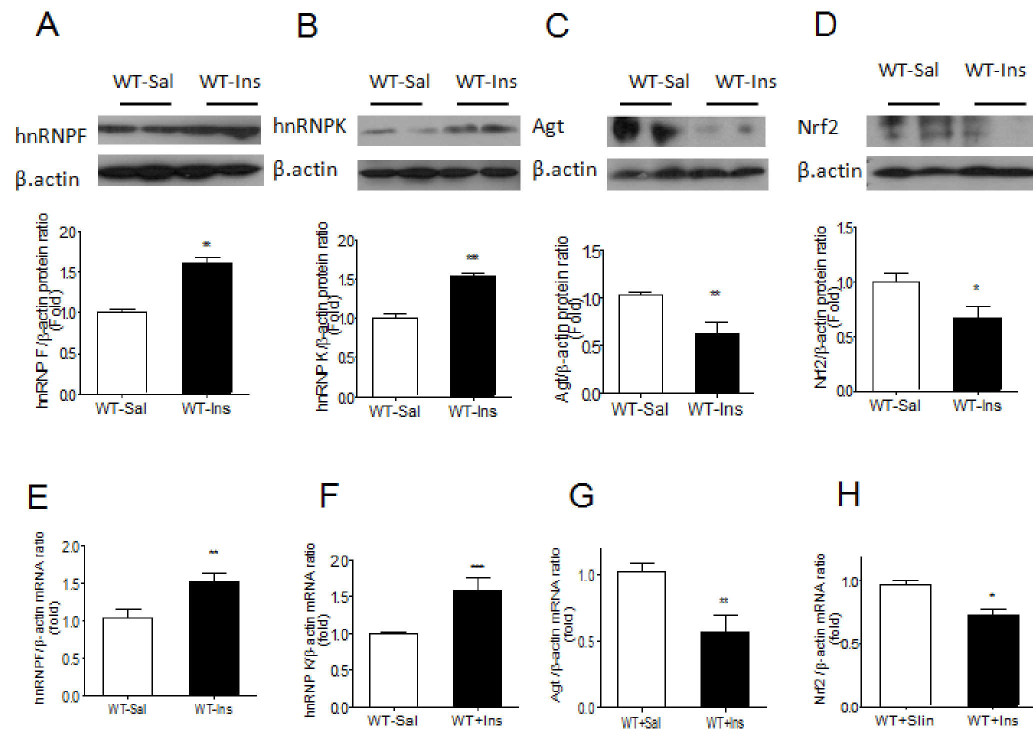


Figure 5-2; HnRNP F, hnRNP K, Agt and Nrf2 gene expression in hyperinsulinemic-euglycemic experiment; A,B,C and D shows the protein level of *hnRNP F*, *hnRNP K* , Angiotensinogen and *Nrf2* respectively. E, F, G and H show the *hnRNP F*, *hnRNP K*, Angiotensinogen and *Nrf2* mRNA expression by real time PCR. Animal number 7 per group. Protein antibodies ; hnRNP F titre(1 :5000), *hnRNP K*(1 :1000) cat #sc-28380, *Agt* (1 :2000) and *Nrf2* (1 :1000).

5.2 Generation of hnRNP K transgenic mice

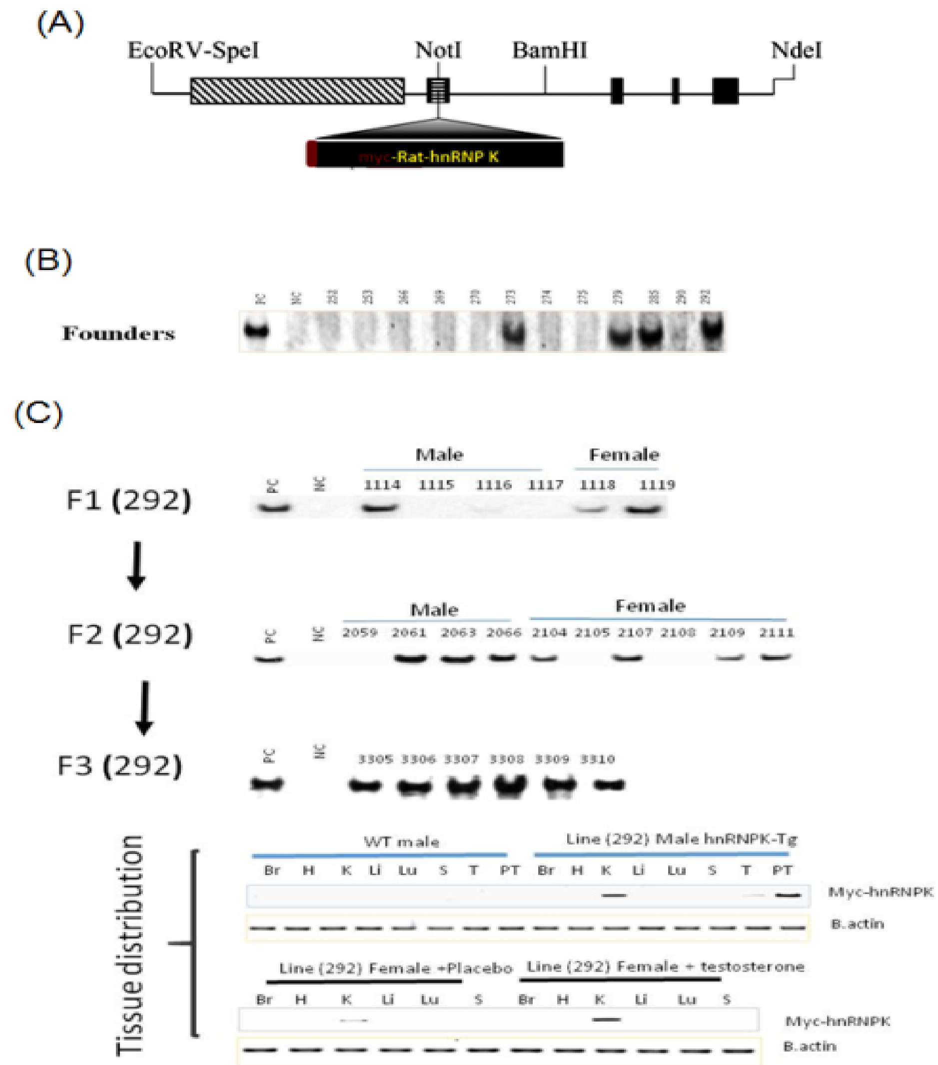


Figure 5-3: Generation of hnRNP K-Tg. A: Schematic map of the KAP2-rat hnRNP K construct. The isolated 17-kb KAP2-myc-hnRNP K transgene (digested with SpeI and NdeI) was microinjected into one-cell fertilized mouse embryos obtained from superovulated C57Bl6 3 C3H mice (performed at the Clinical Research Institutes of Montreal, Montreal, Quebec, Canada). B: Southern blotting of genomic DNA to detect for founders with radioactive *hnRNP F*probe. C: Heterozygous and homozygous F1, F2, and F3 were screened by PCR with specific primers. RT-PCR product showing tissue expression of *myc-hnRNP K* mRNA in non - transgenic, male-Tg and female-Tg with and without testosterone. b-Actin *myc-hnRNP K* fragments are indicated. Br, brain; H, heart; K, kidney; Li, liver; Lu, lung; S, spleen; T, testis.

Immunohistochemical (IHC) staining was performed according to the standard avidin–biotin–peroxidase complex method (ABC Staining System, Santa Cruz Biotechnologies, Santa Cruz, CA, USA). Renal sections ofWT mice and *hnRNP K* –Tg mice were incubated with non-immune serum (1:100 dilution) or primary anti-*hnRNPK* (1:100 dilution). IHC staining showsan increase of hnRNP K protein in the RPTCs of male Tg mice in comparison with male wild type (WT) mice. Freshly isolated RPTs from wild type and *hnRNP K*-Tg used for W.B. analysis. *HnRNP K* is overexpressed in the RPTs of transgenic mice compared with non transgenic, Figure 4-4.

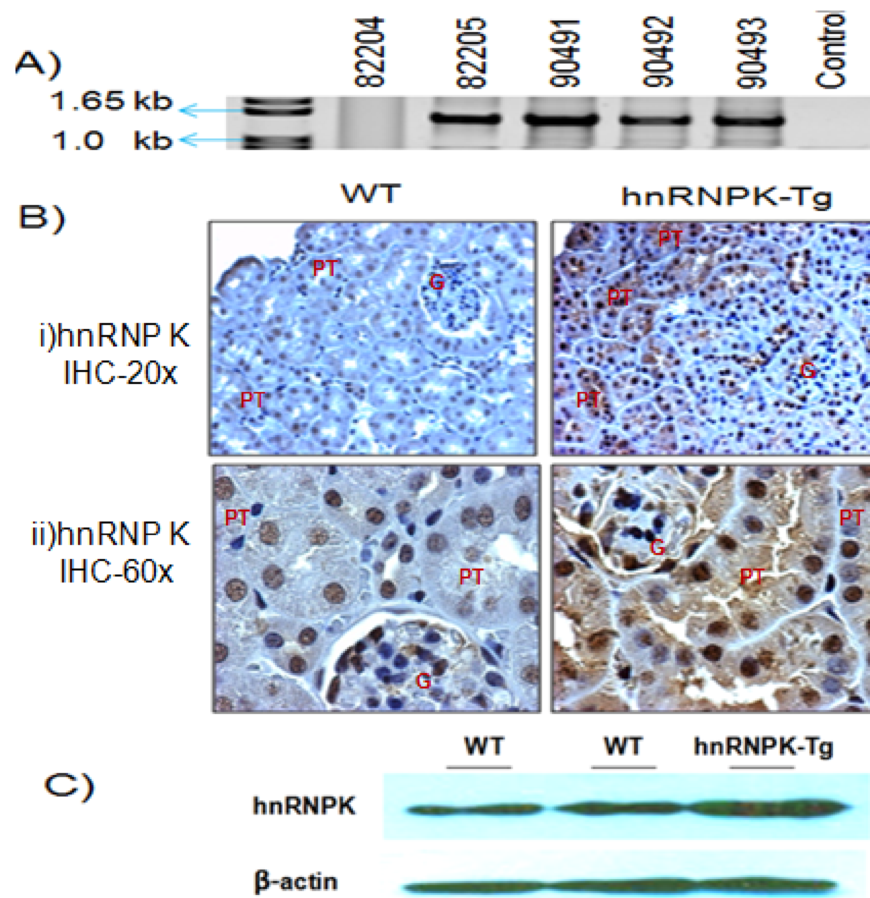


Figure 5-4 : Characterization of *myc-hnRNP K*-Tg mice. A; DNA genotyping for *hnRNPK*-Tg. B; IHC for WT mouse and *hnRNPK*-Tg using *hnRNPK* antibody. C; W.B analysis for WT and *hnRNPK*-Tg mouse using *hnRNPK* antibody (cat # sc-28380**)** titre 1 :1000 and IHC titre 1 :150. Mice number 2 per group.

To assess the abnormalities in kidney structure and collagenous components in *hnRNP K*-Tg mice, we performed periodic acid-Schiff (PAS) and Masson's trichrome staining for kidney sections (4–5 sections, 3–4 mm thick, per kidney) of WT mice and *hnRNP K*-Tg mice. Staining with PAS demonstrates normal basement membranes in *hnRNP K*-Tg mice. Masson's Trichrome shows no significant differences in the expression of collagenous proteins between the WT and the transgenic mice. These observations suggest that overexpression of *hnRNP K* in RPTs does not cause renal damage at the morphological level (Figure 4-5)

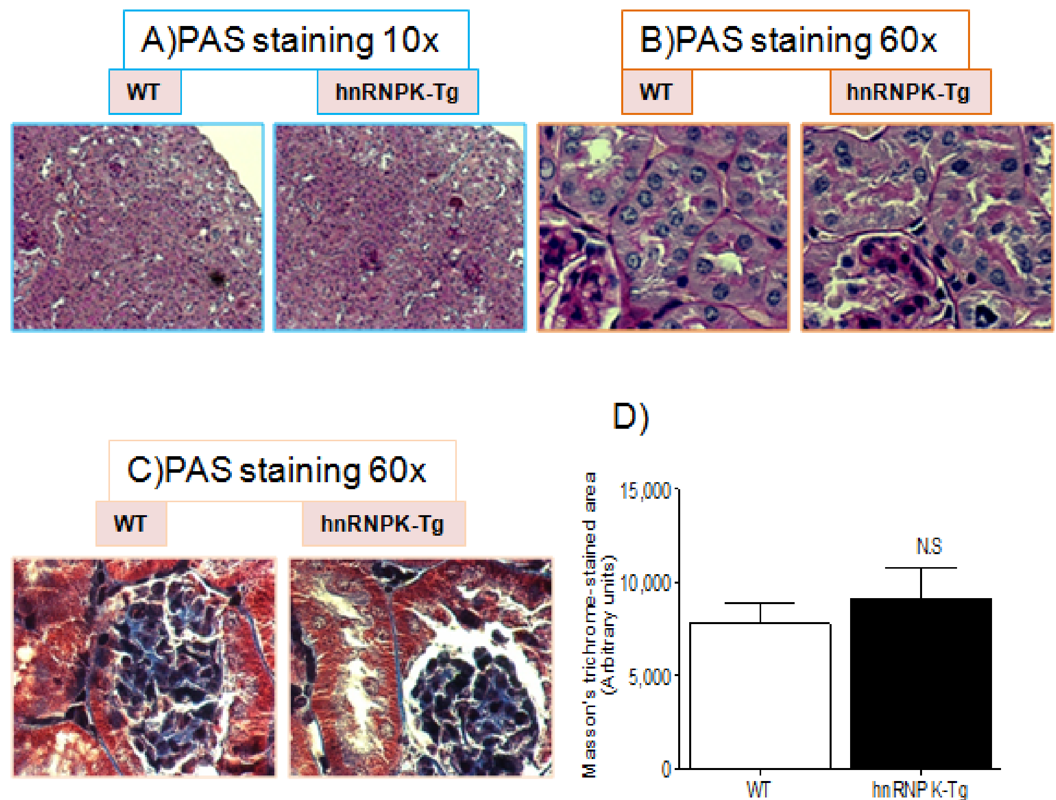


Figure 5-5 : Effect of hnRNP K overexpression on kidney structure and glomerulotubular fibrosis. A and B; Periodic acid-Schiff (PAS) staining (10x &60X) C; Masson's Trichrome staining of collagenous components expression in mouse kidneys D ; quantification of extracellular matrix component accumulation (Masson's trichrome staining) using ImageJ software: WT control and *hnRNP K*-Tg mice (mice number 2 per group).

To test whether overexpression of *hnRNP K* in the RPTs of diabetic mice will either rescue or prevent kidney injury or hypertension, we bred male heterozygous Akita mice with homozygous female *hnRNP K*-Tg mice to produce Akita-*hnRNPK*-Tg mice. These hybrids are identified by PCR for the mutated insulin gene 2 and for *KAP- hnRNPK* transgene expression.

To study the effect of the *hnRNP K* transgene in the Akita mouse model, the male mice were divided into 4 groups: 1. Non transgenic mice (controls) 2. *Kap2-hnRNPK*-Tg mice 3. Akita mice and Akita- *hnRNPK*-Tg mice. Body and kidney weights were recorded. The animals sacrificed after measuring blood pressure, blood glucose, collecting urine and measuring GFR. Trunk blood was collected for plasma *Agt* and ANG II analysis. The left kidney of the mouse was removed and processed immediately by histology and immunohistochemistry. The preliminary data for 5 mice in each group are summarized in fig 5-6, showing a potential role of *hnRNPK* overexpression in the RPTs of Akita mice on kidney size and blood pressure without affecting blood glycemia. The blood pressure was measured using the tail cuff method by Abouzar Otoukesh, Anindya Ghosh, and Ranjit Singh (students of Prof.Dr. Chan's lab). More experiments need to be performed in order to show the histology of the kidneys in the four groups, as well as quantification of *Agt* gene expression by quantitative RT-PCR and western blot. In addition to the aforementioned genes, it would be preferable to study the effects of *hnRNPK* expression on all the RAS system genes including *ACE, ACE2, AT1R, AT2R, MAS* receptors as well as renin, including microarray screening for certain genes. Since *hnRNPK* plays an important role in alternative splicing, apoptosis should be assessed as well.

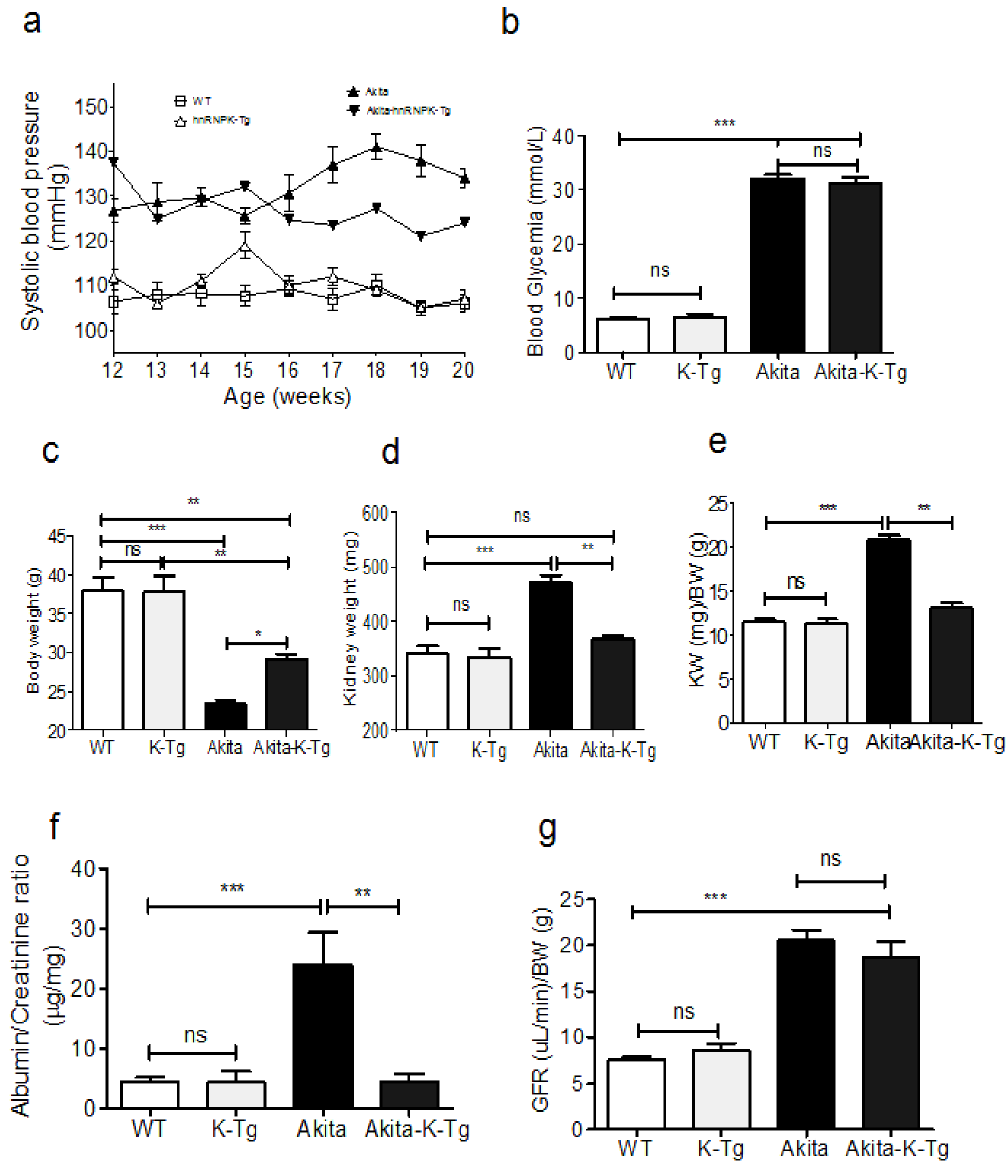


Figure 5-6 Overexpression of *hnRNP K* attenuates systemic hypertension in Akita Tg mice. (a) Longitudinal changes in mean SBP (tail cuff system) in male WT control (□), *hnRNP K*-Tg (△), Akita (▲), and Akita *hnRNP K*-Tg mice (▼). The mice were trained in the procedure for at least 15–20 min per day for 5 days before the first SBP measurements. Blood glycemia level (b) body weight (c) kidney weight (d) andbody weight/kidney weight ratio (e).Urinary albumin/creatinine ratio (f) and glomerular filtration rat (GFR) (g).All data are expressed as a mean of 5 mice per group WT mice empty bars,*hnRNP K*-Tg mice light gray bars, Akita mice black bars and akita-*hnRNP K*-Tg mice dark gray bars. *P < 0.05; **P < 0.01; ***P < 0.00.

5.3 Rat *hnRNP F* and *hnRNP K* gene promoters.

The rat *hnRNP F* gene promoter (N-1500 to N+99) and the rat *hnRNP K* gene promoter (N-1516 to N+16) were cloned from respective rat genomic DNA by conventional polymerase chain reaction (PCR) with specific primers. HnRNP F primers are; F 5'-aaa ggt acc ttt tta aag tct taa gca ttt g-3' and R: 5' aaa aag ctt cag ggg aaa cgc ttt tcg-3' whereas hnRNP K primers are F: 5' aaa ggt acc gga ggc aac ggc gga ctc gc-3' and R: 5'-aaa aag ctt acc aat tca cca ttg gtt tcg g-3'. The promoter inserted into pGL4.20 vector via Kpn I and Hind III restriction sites. Rat *hnRNPF* promoter analysis is shown in Figure 5-7 and rat *hnRNP K* in Figure 5-8.

Rat *hnRNP F* promoter sequence (1600 nucleotides); NCBI Reference Sequence: NC_005103.3

```
-1500 tttttaaagtcttaagcatttgcagcttatcaccagctttagtcaatagtgggccattttgtttcatgtttctctccaggttttgcaaaaaaaaaaaaa
-1400 aacttagttacaacatattttaaagtatttgtatagtagtgcttgccggggttttctctacaggtgcttaggtcagagaacaacttttgccctttctctt
-1300 ctgtgggtctgtggatggttctccactcaggtgtcaaacaagctaggtgactagtgccttgccccatttttctgggccctcaaaaaaaaatgagaagtg
-1200 actggattatttcagaggccaattgctttgaattaagatttgtggttgcctgttgggtgtagattttataaggttcatttttaaaatatttatttaacg
-1100 tatatgagtacattgtagctgtcttgagatacaccagaatctgttagaaatggttgtgagccaccgcgtggttgctgggaattgaactcagtgctcttaa
                                 GATA
-1000 ctgctgagccatctctccagcccctataaggttcattttatacatatggccaacttaggtttaaaagtcaagcactccttccactacggcgatactag
                                                                                                GATA
-900  ggatccaaacctagtcagcctctagggctctgagggaactcgagtagtcccggcaggtctcgaaatcagcttggctctgcctccagagtgatggtattaa
-800  aaaaatgtatgtaccataccggtcattagtttatgtaaaaaaatttgagacaaggctaaatgatgggattaaaggcgtgccgccaccaagtcgagtcggg
-700  agtttttaaaagttaggttatcgtggagtcggcgggcaaatgaaaaaagtgtagagtgtctgcaggtttccattctgtactccgagttgatttggtttta
-600  cttggctgagtctgtcaacgcttcgttgcattcgcctttccaactgtagcgggtagtctgtactttgctcaggtggctcctttacccagaactcagcgag
-500  gagtcttcccctttcctttccctccttcccccacttccggcgccggccaaacccaccgttcgcgggccacacctgcaggcacgcttttctcattggtgac
                                                                                                   CRE
-400  atattggcgggcccggcagggcacttcctcctctacaccccgtgacccgagtccgactgccgctgtagatcacgcggctgctgcttcccggactccggac
                                                 CRE
-300  tcgaacgcccgtctcggcgtggtgagccccgcttcccgcccgagccccaggttaccggctgcgtacttgacctgctcaccttagaagtttccctcttcga
                                        Sp1                            CRE
-200  gcttttccacgcttcggcctgcgcgagttcttactccagccctcttccccgccctctggatcttcccctccccccaccctccgaggctcaggagcctccct
                                                        Sp1
-100  tcccccccgcccccgcccggaagctcccgcccgagcccgcgcaagcccccggggcggaccttcctgcccggccggcgctcgctgcgcggcgcggtcgactac
          Sp1   Sp1               Sp1                                                           -1
+1    cgggcgaaggccgcggagcagcgtcaccttgccctccgccctgcttccgccaaaacgctcgtctcaagggctccgcttccgcaaaagcgtttcccctg
      +1TSS
```

Figure 5-7 Rat *hnRNPF* gene promoter sequence and analysis; Tss (Transcription starting site) at +1; Genomatix software suite v2.3 (http://www.genomatix.de) and TFBIND website (http://tfbind.hgc.jp/) analyzed the promoter sequence for transcription starting site and DNA regulatory elements.

Rat *hnRNP K* promoter sequence (1600 nucleotides); NCBI Reference Sequence: NC_005116.3.

```
-1516 ggaggcaacggcggactcgcgcggtgagcaaggccgggcgagccg cgctt gcggga gcggg gccgg acccct agtcggctgtcgtgggggcgctcgggcc
                                CRE
-1416 ggtcatggccggccggggaacgttgcccgggctcccctcccccac cgccg ccattt ccccg agcgt gcctct gcagttggcgtgctggggctagatggtg
-1316 tcaggggccggtggggaaaggcctgtgggcttaggacccggcgc ccagt gtggag ggagc gccgg gctggc cggtgggctcgcgggtgtcgtgcgccgc
-1216 ggcgccccgccgccctctttgtccctccacagcgtgggcctccc tcccc caggtg ggggt gctgc gcggcg ggcggatggggcggccgcggctcccgc
                 sp1  Sp1
-1116 ggctgctttgtctcgggcagtgggggaggtgcgggggacgggcgc tcgcg agcccg cgtgc tgggt ggggga gggggtctcgcgcgactccgcctagtc
                                                                                    palindromic sequence
-1016 gcacgtagtcgccattacgcgggccgccatttcctgtcgggtccg ggtga ggagag cctcg gcgcg ccgctc ggcgacggtagcggggcgcttcgtgggg
-916  ccgggccgtgggcgggaggacgacgcgtgctgtctgtcgctcggc ggacc tgttag tgcta gcggg tttcgg ggaccgcggctacagggcgagggctcga
-816  ctatcttgcgtagaccctggtgctcatttgcttgaacccctttgc cctcg cagctg gagct tgagc agagta accgggctcggcgtttcaagtcaccgtc
                                                                     CRE
-716  agggtgacagacacttgcagccggcgttttggaataggagaagtc ataag tgcgat acccg tgata ttctgt gctagtccttggtttggtaggaccacaa
          CRE                                                   GATA        GATA
-616  gttgaccggaccagttgtagcgctttggttgcagtcttggagagt tgaat aagagg cgaaa ttgtt gggagg cagcctttctaacatcacttaaaatagt
-516  ggtgctggtggttaaaggctccgtggctttgcgataactgtggtagtaat atcaat ctggt gcctt aatcag aatttagatttctctgtgtgaaagttt
                                     GATA
-416  aatgtagtttgtatttgccgttaggttttgtgaggttggcagatt taatt gattag tgtca gtcac gacagg ggtgggggcacaagtaactactagtcct
                                    CRE
-316  agctttgtgataagacttggtcttttcaagaattagtcttgtgaacaaaa ttttaa gtcgg ctggc ttttcc gttgt aatgaaatgaagcaagttgctga
              GATA
-216  ggacagaattactgatgtttatttctttgggggggaggttttg gtggt ggaagt ggtta gactt ttgttt cattgagacagtctgaatagtctaaaag
                                                                                     CRE
-116  gaatccattacacctgtctctcaatttgtatagttttaaaaattgtaaa caaaat agtct ttgct ttctga taagccaaatatacaagctagcttcttt
                                                                                GATA
-16t  atttttaacagattggtggatccatttagttcaagaaaatggag accga acagcc agaag aaacc ttccct aacaccgaaaccaatggtgaatttggt
                    +1TSS
```

Figure 5-8 Rat *hnRNPF* gene promoter sequence and analysis Tss (Transcription starting site) at+1 Genomatix software suite v2.3 (http://www.genomatix.de) and TFBIND website (http://tfbind.hgc.jp/) analyzed the promoter sequence for transcription starting site and DNA regulatory elements.

The plasmids pGL4.20- *rhnRNP F*-N-1500 - N+99 and r*hnRNP K* -N-1516 to N+16 were stably transfected into rRPTCs.To study the effects of HG and insulin*, hnRNP F* and *hnRNP K* stable transformant cells at 75-85% confluency were synchronized overnight in serum-free DMEM containing 5 mmol/l D-glucose, then incubated in medium containing 5 mmol/l D-glucose plus insulin in different doses or 5mmol/l D-glucose plus 20 mmol/l D-mannitol (normal glucose) in the absence or presence of HG (25 mmol/l D-glucose) with and without insulin in DMEM containing 1% depleted FBS for 24 h,Figure 5-8.

a b

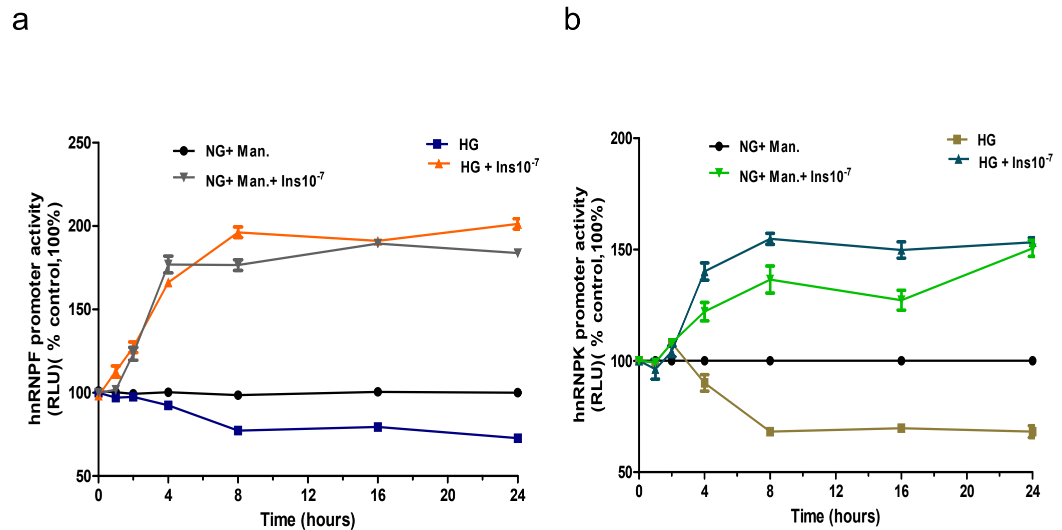


Figure 5-9 shows *hnRNP F* and *hnRNP K* gene promoter activity in normal glucose (5mmol/l D-glucose plus 20 mmol/l D-mannitol) and high glucose 25 mmol/l D-glucose) with and without insulin 10^{-7}.

5-4 Transcription factors and the angiotensinogen promoter.

The angiotensinogen gene promoter has an IRE binding site as well as *Nrf2* binding site. Our data demonstrate that high glucose downregulates *hnRNP F* and *hnRNP K*and increases *Nrf2* and angiotensinogen gene expression. We would like to investigate the competition between the three transcription factors on the *Agt* gene

promoter. Using the *Agt* promoter stable clone we will transfect each transcription factor in combination with *Nrf2* to elucidate the major effector on *Agt* promoter activity.

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