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ANTIAPOPTOTIC EFFECT OF OUABAIN IN RENAL CELLS EXPOSED TO TOXIC GLUCOSE LEVELS

Liang Zhang
张良

Karolinska Institutet

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Antiapoptotic effect of ouabain in renal cells exposed to toxic glucose levels
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Liang Zhang

Principal Supervisor:
Lena Scott, Ph.D.
Karolinska Institutet
Department of Women's and Children's Health
Division of Pediatric Cell and Molecular Biology

Co-supervisor(s):
Senior Professor Anita Aperia
Karolinska Institutet
Department of Women's and Children's Health
Division of Pediatric Cell and Molecular Biology

Jacopo Maria Fontana, Ph.D.
Royal Institute of Technology
Department of Applied Physics

Opponent:
Adjunct Associate Professor Sandrine Pierre
Marshall University
Department of Pharmacology, Physiology and Toxicology

Examination Board:
Professor Emerita Barbara Cannon
Stockholm University
Department of Molecular Biosciences

Docent Alexander Chibalin
Karolinska Institutet
Department of Molecular Medicine and Surgery

Professor Boris Zhivotovsky
Karolinska Institutet
Institute of Environmental Medicine

ABSTRACT

Diabetes mellitus is a group of metabolic diseases that causes enormous social and economic burden. The kidney is the filtration organ that produces urine and is the primary target of diabetic complications, a condition called diabetic nephropathy (DN). DN is a form of chronic kidney disease (CKD) and CKD is characterized by a progressive decrease in renal function which results in renal failure. Diabetes mellitus is characterized by hyperglycemia which induces renal cell apoptosis and causes tissue loss in the kidney. Together, these hallmarks are the major pathophysiological events observed in DN. Bcl-2 family proteins play an important role in the intrinsic apoptosis pathway and the family member, BAD, has been shown to be proapototic. Ouabain is a cardiotonic steroid and a highly specific Na, K-ATPase ligand. The binding of ouabain to Na, K-ATPase has been suggested to activate downstream signaling. Ouabain-bound Na, K-ATPase can interact with the 1, 4, 5-trisphosphate receptor, generating a calcium signal that has a protective effect. In this thesis we have studied the global signaling pathways generated by ouabain-bound Na, K-ATPase, and the mechanism responsible for hyperglycemia induced renal cell apoptosis. In addition, we have investigated the molecular mechanism behind ouabain's protective effect against apoptosis.

In study I, we investigated changes in the cellular phosphoproteome after ouabain treatment. A total of 2580 ouabain-regulated phosphorylation events were identified. Among the proteins that were phospho-regulated, a large proportion were involved in the regulation of cell adhesion and proliferation. Multiple protein kinases, including Ca^{2+}/calmodulin-dependent protein kinase were also regulated. This study revealed that Na, K-ATPase is a versatile signal transducer of its ligand, ouabain.

In study II, we used primary cell cultures of proximal tubular cells (PTCs), podocytes and mesangial cells to study their sensitivity to high glucose treatment. We found that PTCs and mesangial cells were more vulnerable to glucose toxicity than podocytes due to their expression of sodium-dependent glucose cotransporters (SGLTs). SGLT inhibition or down-regulation protected renal cells from high glucose induced apoptosis and ouabain treatment had the same protective effect.

In study III, we applied super resolution microscopy to follow the apoptotic process triggered by high glucose. After two hours of high glucose treatment, the proapoptotic protein, BAD, had translocated to the mitochondria and initiated apoptosis. BAD translocation to mitochondria was abolished in the presence of ouabain. This study highlighted BAD's role as an important player in glucose induced apoptosis and demonstrated the protective effect of ouabain which intervenes early in the apoptotic process, by preventing BAD activation.

LIST OF SCIENTIFIC PAPERS

I. Elena Panizza, **Liang Zhang**, Jacopo Maria Fontana, Kozo Hamada, Daniel Svensson, Evgeny E. Akkuratov, Lena Scott, Katsuhiko Mikoshiba, Hjalmar Brismar, Janne Lehtio, and Anita Aperia.
Ouabain-regulated phosphoproteome reveals molecular mechanisms for Na^+, K^+-ATPase control of cell adhesion, proliferation, and survival. FASEB journal: official publication of the Federation of American Societies for Experimental Biology. 2019; 33(9):10193-206.

II. Linnéa M. Nilsson, **Liang Zhang**, Alexander Bondar, Daniel Svensson, Annika Wernerson, Hjalmar Brismar, Lena Scott, and Anita Aperia.
Prompt apoptotic response to high glucose in SGLT-expressing renal cells. American journal of physiology Renal physiology. 2019;316(5):F1078-F89.

III. **Liang Zhang***, Daniel Svensson*, Jacopo Maria Fontana, Kristoffer Bernhem, Linnea M. Nilsson, Lena Scott, Hans Blom, Hjalmar Brismar and Anita Aperia.
Mapping of glucose induced apoptosis in kidney epithelial cells and the anti-apoptotic effect of ouabain - a BAD story. Manuscript.

* Contributed equally

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LIST OF ABBREVIATIONS

2-NBDG	2-[N-(7-Nitrobenz-2-oxa-1, 3-diazol-4-yl) amino]-2-deoxy-D-glucose
ACE	angiotensin converting enzyme
ACTH	adrenocorticotrophic hormone
AGEs	advanced glycation end products
ALS	amyotrophic lateral sclerosis
ARB	angiotensin II receptor blocker
CaMK	Ca^{2+}/calmodulin-dependent protein kinase
CaMKK1	Ca^{2+}/calmodulin-dependent protein kinase kinase 1
CAPOS	Cerebellar ataxia, areflexia, pes cavus, optic atrophy, sensorineural hearing loss
CTS	cardiotonic steroid
DCFH-DA	2', 7'-Dichlorodihydrofluorescein Diacetate
DKD	diabetic kidney disease
DN	diabetic nephropathy
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
EGFR	epidermal growth factor receptor
EO	endogenous ouabain
ESRD	end-stage renal disease
ETC	electron transport chain
FasL	Fas ligand
FHM	Familial Hemiplegic Migraine
FPALM	fluorescence photoactivation localization microscopy
FRET	Förster resonance energy transfer
GBM	glomerular basement membrane
GEC	glomerular endothelial cell
GECI	genetically encoded calcium indicator
GFR	glomerular filtration rate
GLP-1	glucagon-like peptide-1

HiRIEF	high-resolution isoelectric focusing
iDPP4	dipeptidyl peptidase-4 inhibitors
IMM	inner mitochondrial membrane
IP3R	1,4,5-trisphosphate receptor
LC-MS	liquid -chromatography-MS
MAPK	mitogen-activated protein kinase
MI	myocardial infarction
MLKL	mixed-lineage kinase domain-like
MPT	mitochondrial permeability transition
MS	mass spectrometry
OLC	ouabain-like compound
OMM	outer mitochondrial membrane
OXPHOS	oxidative phosphorylation
PALM	photoactivated localization microscopy
PCD	programmed cell death
PCR	polymerase chain reaction
PEC	parietal epithelial cell
PKC	protein kinase C
PTC	proximal tubular cell
RDP	Rapid-onset Dystonia Parkinsonism
RIPK	Receptor-interacting serine/threonine-protein kinase
ROS	reactive oxygen species
SBFI	Na^+-binding benzofuran isophthalate
SIM	structured-illumination microscopy
siRNA	short interfering RNA
STED	stimulated emission depletion microscopy
STIM1	stromal interaction molecule 1
STORM	stochastic optical reconstruction microscopy
TCA	tricarboxylic acid
TM	transmembrane

TNF	tumor necrosis factor
TNFR1	TNF receptor 1
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
TZD	thiazolidinedione
VDAC	voltage-dependent anion channel
WHO	World Health Organization

1 Introduction

1.1 The kidney

1.1.1 Kidney physiology

The primary functions of mammalian kidney include filtration of wastes, maintenance of essential solutes, erythropoiesis, and regulation of blood pressure, water and electrolytes, the balance of acid-base and calcium and phosphate (Wallace, 1998). In a healthy human, the kidneys filter about 130 ml or 120 ml blood volume per minute per 1.73 m^2 in young man and woman respectively (Stevens, Coresh, Greene, & Levey, 2006) generating approximately 1 liter of urine per day. The functional unit of the kidney is the nephron, which is composed of a glomerulus and tubular system, and an adult kidney has an average of 1 million nephrons. The glomerulus, which is responsible for blood filtration and metabolic waste concentration, has four types of cells: podocyte, mesangial cell, glomerular endothelial cell (GEC) and parietal epithelial cell (PEC). The podocytes, GECs and their shared extracellular matrix (glomerular basement membrane, GBM) form the filtration barrier to filter blood generating the primary urine. The primary urine flows to the tubular system for further reabsorption and regulation of the components of urine (Scott & Quaggin, 2015). The reabsorption of glucose takes place in proximal tubule cells (Wood & Trayhurn, 2003). Figure 1 shows an anatomical overview of renal filtration.

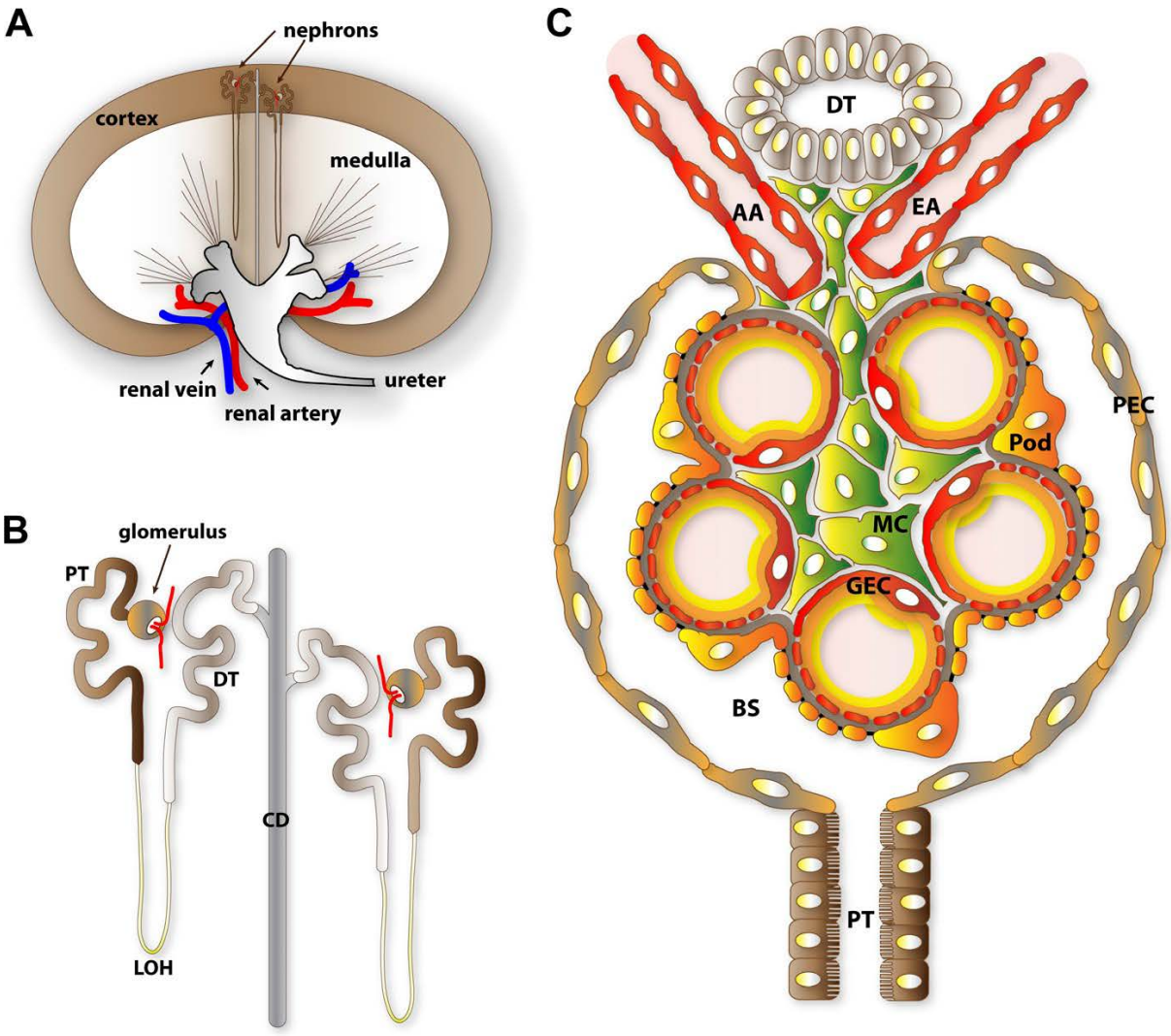

Figure 1: overview of renal filtration (A) Schematic shows that the nephrons are distributed in the kidney cortex. (B) Structure of the nephron, which is composed of a glomerulus and tubular system including proximal tubule, loop of Henle, distal tubule and collecting duct. (C) Structure of the glomeruli. The podocytes, GECs and their extracellular matrix form the filtration barrier.

PT, proximal tubule; LOH, loop of Henle; DT, distal tubule; CD, collecting duct; AA, afferent arteriole; EA, efferent arteriole; GEC, glomerular endothelial cell; Pod, podocyte; MC, mesangial cell; PEC, parietal epithelial cell; BS, Bowman's space. Figure is reprinted from (Scott & Quaggin, 2015).

1.1.2 Renal cells and their glucose transporters

Glucose is a key fuel and metabolic substrate in mammals. The glucose is transported across the plasma membrane to be utilized in the cell or to be absorbed into the body, as in the luminal membranes of cells in small intestines and proximal tubules of the kidney (Wood & Trayhurn, 2003). There are two major types of glucose transporters expressed in renal cells: the facilitative glucose transporters (GLUTs) and sodium-dependent glucose cotransporters (SGLTs). GLUTs utilize the diffusion gradient of glucose across the plasma membrane, while

the latter are cotransporters of sodium and glucose using the Na^+ gradient created by the Na, K-ATPase to transport glucose into cells against its concentration gradient. The SGLT1, mainly expressed in the small intestine, has high affinity and low capacity (cotransport one glucose molecule with two sodium ions); the SGLT2, expressed dominantly in renal proximal tubules (S1 and S2 segments) has low affinity and high capacity (cotransport one glucose molecule with one sodium ion). SGLT2 is responsible for the absorption of the majority of glucose in the proximal tubule while SGLT1, which is expressed in the S3 segment of proximal tubules, absorb the remaining glucose to prevent glucose loss in the urine (Wright, Loo, & Hirayama, 2011).

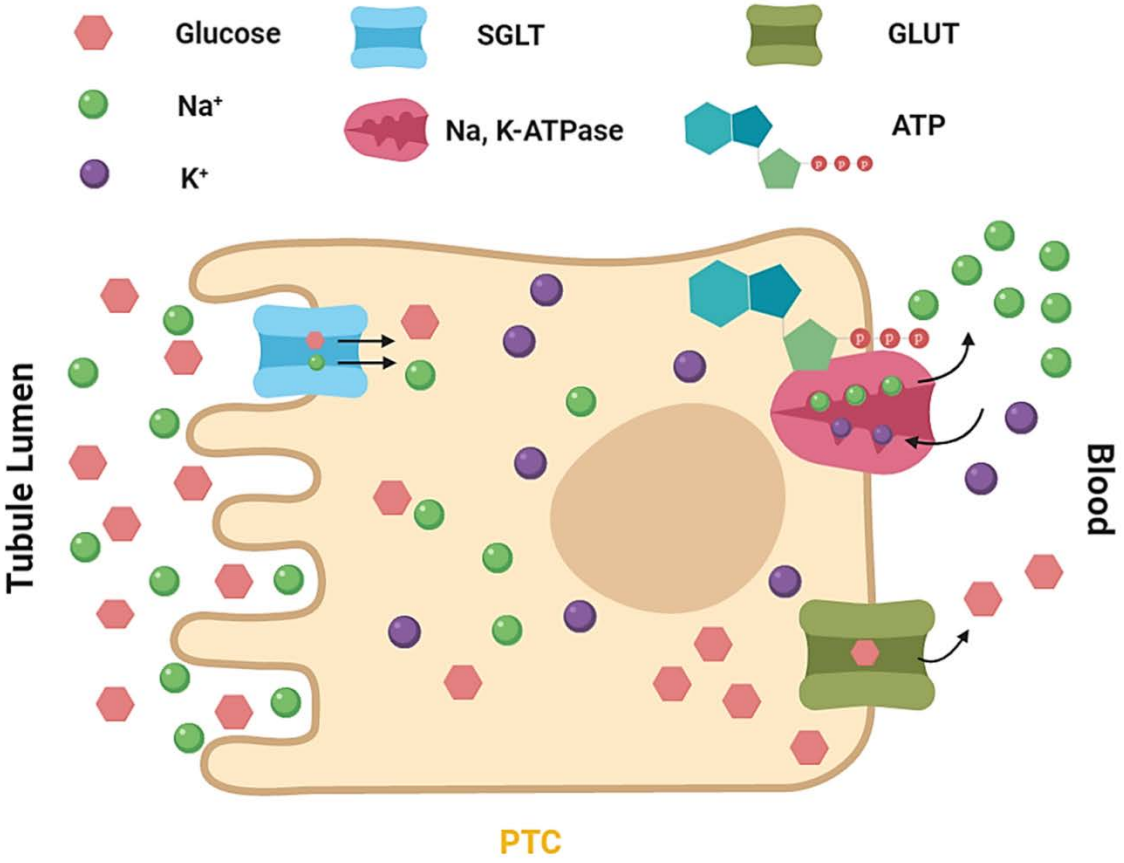

Figure 2: Glucose transport in proximal tubular cell (PTC). On the apical side, SGLTs cotransport glucose and Na^+ from the tubule lumen to the cell. On the basolateral side, GLUTs transport glucose back to interstitial space and finally blood stream. The Na, K-ATPase transports 3 Na^+ out and 2 K^+ into the cell using one ATP molecule, creating the Na^+ gradient that drives SGLTs. The figure is designed using Biorender online tool: https://biorender.com/.

1.1.3 Chronic kidney disease and current treatments

Chronic kidney disease

According to The Kidney Disease: Improving Global Outcomes (KDIGO) 2012 clinical practice guideline, the definition of chronic kidney disease (CKD) is: decreased renal function shown by kidney damage markers or a glomerular filtration rate (GFR) of less than 60 ml/min/1.73 m^2, or both, for more than three months (Levin & Stevens, 2014). The prevalence of CKD is around 11% in America and Australia for the population in general, however, people with lower socioeconomic status have 60% more chance of having CKD. Diabetes accounts for 30-50% of all CKD and affects 6.4% of the adult population worldwide (Webster, Nagler, Morton, & Masson, 2017).

Current treatment

Lifestyle and dietary change is recommended, examples are exercise, smoking cessation and decreased alcohol intake. In patients with hypertension, low sodium consumption is recommended and most patients with CKD should avoid high-protein diets. Blood pressure and glycemic control is needed when CKD patients have hypertension and diabetes. Angiotensin converting enzyme (ACE) inhibitors and angiotensin II receptor blockers (ARBs) are beneficial even to CKD patient without hypertension. In spite of these available treatments, CKD is a progressive disease and results in end-stage renal disease (ESRD). In America alone, there are half a million people who have ESRD. Patients with ESRD need hemodialysis or peritoneal dialysis and optimally kidney transplantation (Drawz & Rahman, 2015).

1.1.4 Diabetic kidney disease

Diabetes is the primary cause of CKD in the world. CKD caused by diabetes, which is called diabetic kidney disease (DKD) or diabetic nephropathy (DN), is accountable for most cases of ESRD, with the number being over 50% in some countries (Sifuentes-Franco, Padilla-Tejeda, Carrillo-Ibarra, & Miranda-Diaz, 2018). DN is also accountable for the major morbidity and mortality in diabetic patients. DN is characterized by glomerular and tubular hypertrophy, thickening of the GBM at the early stage and tubulointerstitial fibrosis and glomerulosclerosis as the disease progresses, which are responsible for the loss of renal tissue (Wolf & Ziyadeh, 1997).

Apoptosis, a programmed cell death, has been suggested as the mechanism behind renal tissue loss. First of all, compared to control samples, kidney biopsies from a variety of experimental animals with kidney diseases and DN patients had more presence of apoptosis (Kumar, Robertson, & Burns, 2004; Woo, 1995). The expression of pro-apoptotic genes were also upregulated in diabetic kidneys and proximal tubular cells (PTCs) exposed to high glucose (Ortiz, Ziyadeh, & Neilson, 1997). Furthermore, high glucose can cause apoptosis in PTCs by inducing oxidative stress and high glucose-induced apoptosis was considered as the main cause for diabetic complications (Allen, Harwood, Varagunam, Raftery, & Yaqoob, 2003; Allen, Yaqoob, & Harwood, 2005).

1.2 Diabetes mellitus

Diabetes mellitus, or diabetes, is a group of metabolic diseases characterized by hyperglycemia. According to World Health Organization (WHO), there were around 422 million adults with diabetes in 2014 globally. Diabetes was the eighth leading cause of death in 2012 with 1.5 million people dying from diabetes or its direct complications (Organization, 2016). It is estimated that the number of diabetic patients aged between 20 and 79 years will reach 642 million by 2040 (Ogurtsova et al., 2017).

Diabetes can be classified into 4 categories:

1. Type 1 diabetes (characterized by insulin deficiency)

2. Type 2 diabetes (characterized by insulin resistance)

3. Gestational diabetes (diabetes diagnosed during pregnancy without previous onset)

4. Specific types of diabetes due to other causes, such as genetic defects and drug (American Diabetes, 2019a).

The persistent hyperglycemia in diabetes results in complications in different organs, especially the eyes, kidneys, nervous system, heart, and blood vessels. The tissue damage occurring in these organs is due to a particular subsets of cells are more vulnerable to hyperglycemia. These cells usually lack a negative feedback to reduce glucose transport inside the cell when exposed to hyperglycemia (Brownlee, 2005). The molecular mechanisms behind glucose-induced tissue damage are: polyol pathway, formation of advanced glycation end products (AGEs), activation of protein kinase C (PKC) isoforms and increased hexosamine pathway activity. The common upstream event of these four pathways is the overproduction of reactive oxygen species (ROS) in cells as hyperglycemia increase superoxide production in the mitochondria (Brownlee, 2005).

1.2.1 Type 1 diabetes

Type 1 diabetes is characterized by insulin deficiency caused by autoimmune-medicated destruction of β-cells in the pancreas. Type 1 diabetes is the most common chronic disease among children worldwide. (P. Zimmet, Alberti, & Shaw, 2001). However, the symptomatic onset of type 1 diabetes can be at any age. The diagnostic hallmarks of type 1 diabetes are the classic trio of symptoms (polyuria, polyphagia and polydipsia) and overt hyperglycemia, while the trio of symptoms is more apparent in children and adolescents than in adults. Type 1 diabetes is a polygenetic disease with many known loci responsible for its susceptibility and

patients need lifetime exogenous insulin replacement (Atkinson, Eisenbarth, & Michels, 2014).

1.2.2 Type 2 diabetes

Type 2 diabetes occurs due to insulin resistance with or without abnormal insulin secretion. Insulin resistance can occur in liver, adipose tissue and skeletal muscles. Over 90% of all global diabetes cases are type 2 diabetes and there is an increasing prevalence of it among adolescents and children, making type 2 diabetes the dominant form among this population in the near future (P. Zimmet et al., 2001). Type 2 diabetes may have genetic predisposition, however, it is the change of life style (sedentary life style) and behaviors (unhealthy diet) that are responsible for the increasing epidemic of diabetes. Besides diet and change of life style, type 2 diabetes patients are treated with hypoglycemic agents and may not be dependent on insulin (P. Zimmet et al., 2001).

1.2.3 Complications and medications

Diabetes can cause macrovascular complications, like cardiovascular diseases and microvascular complications, such as retinopathy, neuropathy and DN (Sifuentes-Franco, Padilla-Tejeda, Carrillo-Ibarra, & Miranda-Diaz, 2018). Patients with type 1 diabetes have 10 times higher risk to develop cardiovascular events than non-diabetic population at the same age group (Atkinson et al., 2014), and type 1 diabetes is also accompanied with higher mortality and morbidity after myocardial infarction (MI) (Gottumukkala et al., 2012). Microvascular and macrovascular complications occur in 50% and 27% respectively in type 2 diabetic patients according to a study involving 28 countries (Zheng, Ley, & Hu, 2018). The relative risk for macrovascular and microvascular complications is 2-4 times higher and 10-20 times higher respectively in type 2 diabetic patients than people without diabetes (Zheng et al., 2018).

Among diabetic patients, patients with type 2 diabetes have 40% higher prevalence of CKD than type 1 diabetic patients, as well as a higher risk for CKD development in the future (Cressman et al., 2018). This may be explained by that among type 1 diabetic patients, hyperglycemia is the major cause of nephropathy because of the early onset of hyperglycemia. While patients with type 2 diabetes have other underlining mechanisms to impair renal function, such as hypertension, dyslipidemia, smoking, obesity and last but not least, declined renal function related to aging (Ruggenenti & Remuzzi, 2000).

Strict control of glycaemia is the key factor in reducing diabetic complications. Studies have shown that tight glucose control decreased both macrovascular and microvascular complications in type 1 and type 2 diabetes patients. Blood pressure and lipid control were also reported to lower diabetic complications, for example, the HOPE study and MICRO-

HOPE sub-study showed that ACE inhibitor can lower cardiovascular and renal events in diabetes patients (Gerstein et al., 2000; P. Z. Zimmet, 1999).

1.2.3.1 Treatments for type 1 diabetes:

Pharmacological approaches for type 1 diabetes includes insulin and noninsulin treatment. Insulin is essential for type 1 diabetes treatment as there is an absent function of β-cells. Insufficient insulin administration is responsible for hyperglycemia and systematic disturbances of metabolism. Multiple injection and subcutaneous insulin pump provide the best outcome for patients with type 1 diabetes. Noninsulin treatment consists of hypoglycemic drugs such as pramlintide and some of the drugs used to treat type 2 diabetes.

Surgical treatment for type 1 diabetes is pancreas or islet transplantation, and in these cases immunosuppression is need after the surgery (American Diabetes, 2019b).

1.2.3.2 Treatment for type 2 diabetes

The treatments of type 2 diabetes are oral medication, injectable agents and bariatric surgery.

Oral medications include metformin, alpha-glucosidase inhibitors, secretagogues, thiazolidinediones, dipeptidyl peptidase-4 inhibitors (iDPP4) and sodium glucose cotransporter (SGLT) 2 inhibitor. Metformin is the first line drug for type 2 diabetes acting to inhibit gluconeogenesis. Secretagogues can increase insulin secretion from pancreatic beta cells. Alpha-glucosidase inhibitors bind competitively to alpha-glucosidase, affecting carbohydrate digestion and absorption. Thiazolidinediones (TZD) act by increasing glucose metabolism and decreasing gluconeogenesis. iDPP4 can inhibit DPP4 to preserve the activity of incretins, hormones that stimulate insulin secretion. SGLT2 inhibitors inhibit glucose reabsorption in the kidney and thus decrease plasma glucose level.

Injectable agents consist of glucagon-like peptide-1 (GLP-1) receptor agonist and insulin. GLP-1 receptor agonist can decrease glucagon secretion and increase insulin secretion.

Bariatric surgery is suitable for diabetes patients with high BMI (>35 kg/m^2) to achieve weight loss (Marin-Penalver, Martin-Timon, Sevillano-Collantes, & Del Canizo-Gomez, 2016).

1.3 Mitochondria

1.3.1 Mitochondria in metabolism

Mitochondria are double-membraned organelles and one mitochondrion consists of the outer mitochondrial membrane (OMM), the inner mitochondrial membrane (IMM), the intermembrane space (space between the OMM and the IMM), matrix (space within the IMM) and the cristae (formed by infoldings of the IMM). Mitochondria are the powerhouse of the cell and can generate ATP through oxidative phosphorylation (OXPHOS) via the electron transport chain (ETC), which are protein complexes in the IMM. During OXPHOS, mitochondrial membrane potential (Ψm) is created by pumping protons from the matrix to the intermembrane space by protein complexes (Vakifahmetoglu-Norberg, Ouchida, & Norberg, 2017).

Nutrients like lipids, proteins and carbohydrates can be metabolized into products in the tricarboxylic acid cycle (TCA cycle) and these metabolites are used for OXPHOS in mitochondria eventually. Besides being a metabolic hub, mitochondria can regulate biosynthesis of various macromolecules as TCA intermediates can be used as the material (Vakifahmetoglu-Norberg et al., 2017).

1.3.2 Mitochondria and apoptosis

Apoptosis is a programmed cell death that is important for development and normal physiological function in multicellular organisms. Mitochondria play a central role in regulation of intrinsic apoptosis (for more information on intrinsic apoptosis see section 1.4.2.2) via gating cytochrome c release. Cytochrome c is normally localized in the intermembrane space of mitochondria transferring electron from complex III to complex IV under OXPHOS. However, pore formation on the OMM under stressful situations, such as intracellular Ca^{2+} overload, endoplasmic reticulum (ER) stress and overproduction of ROS, can cause the release of cytochrome c proteins into the cytoplasm and thus induce apoptosis. Besides, mitochondria can amplify extrinsic apoptotic pathway and release proteins that are responsible for caspase-independent apoptosis (Vakifahmetoglu-Norberg et al., 2017).

Furthermore, mitochondria participate in Ca^{2+} homeostasis and any disrupted regulation of intracellular Ca^{2+} can induce apoptosis. The proton leakage during OXPHOS can react with oxygen to generate superoxide making mitochondria the main source for intracellular ROS. ROS over-production also leads to apoptosis (Vakifahmetoglu-Norberg et al., 2017).

1.3.3 Mitochondrial dynamics and biogenesis

Mitochondria change their size and shape through continuous ongoing fusion and fission, the so-called “mitochondrial dynamics”. During mitochondrial fusion two mitochondria merge

together forming a larger mitochondrion. Mitochondrial fission is the division of one mitochondrion into two mitochondria. In healthy cells, there is a balance between mitochondrial fission and fusion. Too much mitochondrial fission causes mitochondrial fragmentation and excessive mitochondrial fusion results in a redundant network of mitochondrial tubules (Galvan, Green, & Danesh, 2017).

Mitochondrial biogenesis is the process of increasing mitochondrial functional properties and abundance. Mitochondrial biogenesis makes cell adaptable to the environment and physiological activities, for example, increased mitochondrial biogenesis was seen under physical activity (Jornayvaz & Shulman, 2010). Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) is considered as one of the master regulators of mitochondrial biogenesis. PGC-1α can dock on transcription factors and regulate mitochondrial gene expression; down-regulation of PGC1α is seen in diseases like DN (Galvan et al., 2017).

1.3.4 Mitochondria in the diabetic kidney

The kidney has the second largest number of mitochondria per tissue mass after the heart. Healthy mitochondria are essential for normal renal function (Parikh et al., 2015).

In one clinical study, 13 urine metabolites were found significantly reduced in diabetic patients with CKD compared to healthy controls and 12 of the 13 metabolites were related to mitochondrial metabolism (Sharma et al., 2013). This indicates an overall decrease of mitochondrial function or content in DN patients. Further evidence to support this is that kidney biopsies from DN patients showed less mitochondrial complex IV and mitochondrial DNA (mtDNA). Fragmented mitochondrial together with increased mitochondrial fission were also seen in kidney cells in both type 1 and type 2 diabetic animal models (Sharma, 2017).

1.4 Programmed cell death

According to recommendations from the Nomenclature Committee on Cell Death, the morphological and molecular criteria to define cell death are loss of integrity of the plasma membrane, complete fragmentation of the cell, as well as nucleus and engulfment of dead bodies by adjacent cells (Kroemer et al., 2009).

The term “programmed cell death” (PCD) was brought up more than 50 years ago to describe the phenomenon of muscle lose during metamorphosis in the silkmoth (Lockshin & Williams, 1964). After that, PCD was found in various developing tissues of different species (Clarke & Clarke, 1996). It is generally known now that PCD plays an important role in development, tissue homeostasis and diseases. In 1972, Kerr and his colleagues found the common

morphological changes in cells undergoing controlled self-deletion and named this type of cell death “apoptosis” (Kerr, Wyllie, & Currie, 1972). Later, more types of PCD were discovered like autophagy and necrosis (Tait, Ichim, & Green, 2014).

1.4.1 In development and diseases

The main feature of PCD is that it is “programmed” to happen in a certain place at a certain time. This precise regulation is thus utterly important for development. Figure 2 shows roles of PCD during development: structure sculpting, metamorphosis and removing abnormal cells. Disrupted PCD causes imbalance between cell proliferation and cell death and can result in diseases. Increased PCD is seen in neurodegenerative disorders and toxin-induced liver diseases, and decreased PCD is seen is cancer, autoimmune disorders and viral infections (Thompson, 1995).

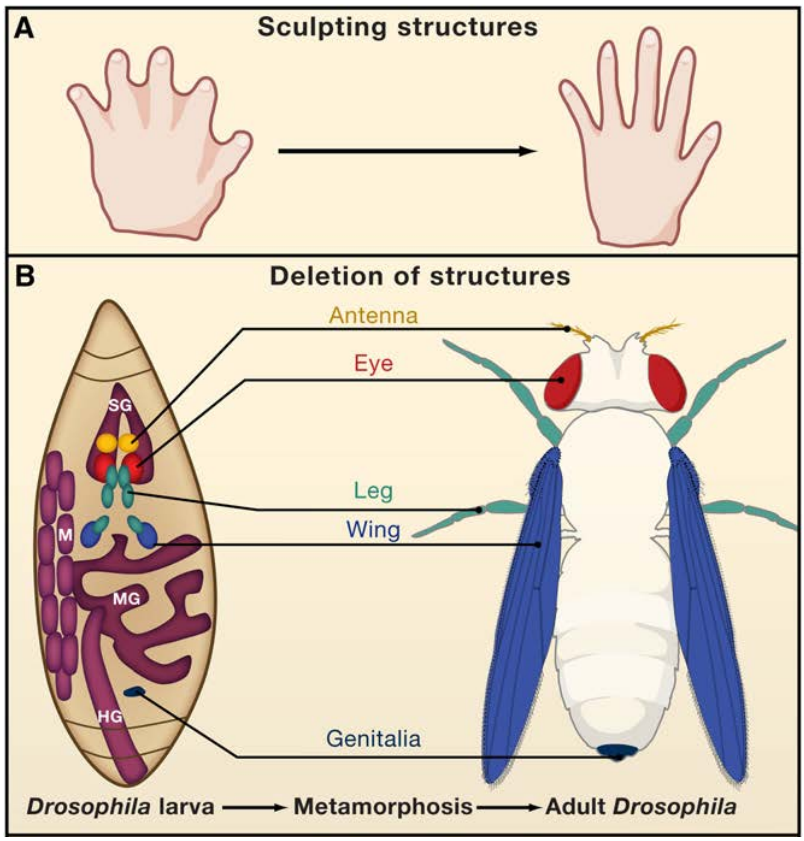

Figure 3: Roles of PCD during development. (A) PCD sculpts the tissue to its proper structure, (B) During Drosophila metamorphosis, the majority of the larval structures are destroyed by PCD. Figure is reprinted from (Fuchs & Steller, 2011).

1.4.2 Apoptosis

In 1972 the term apoptosis was first proposed, Kerr and his colleagues described that the morphological changes of apoptosis happened in two steps: the first one is condensation of nucleus and cytoplasm content and formation of apoptotic bodies from breaking up of the cell; the second step is these apoptotic bodies being eliminated by nearby cells or simply falling away (Kerr et al., 1972). Since then, the molecular mechanism of apoptosis has been explored and different apoptotic pathways has been discovered (Elmore, 2007).

An interesting fact is that “apoptosis” is a Greek word to describe the phenomenon of falling off of leaves or petals. Regarding its pronunciation, Kerr and his colleagues wrote that “the stress should be on the penultimate syllable” and “the second half of the word being pronounced like “ptosis” (with the “p” silent)” (Kerr et al., 1972).

In addition to morphological changes as described above, apoptotic cells share biochemical features: 1. protein cleavage and cross-linking; 2. breakdown of DNA; 3. being recognizable by phagocytes. Protein cleavage is caused by increased caspase activity where the initiator caspases (caspase 2, 8, 9, 10) are activated initially and thereafter regulate executioner caspases (caspase 3, 6, 7). The executioner caspases can cause morphological changes of the cell including shrinkage, DNA condensation, formation of apoptotic bodies and ultimately apoptosis. Apoptosis is therefore the result of a cascade of molecular events (Elmore, 2007).

Classification of apoptosis

The two major apoptotic pathways are intrinsic (the mitochondrial pathway) and extrinsic pathway. There is also a third less common apoptotic pathway called the perforin/granzyme pathway.

1.4.2.1 Intrinsic pathway

Mitochondria play a central role in intrinsic apoptotic pathway and different stimuli can initiate intrinsic apoptosis. One such stimulus is the removal of hormones and growth factors from the cell. When apoptosis was first described, Kerr et al. found that apoptosis was induced in prostate cells after orchiectomy and in the adrenal cortex after withdrawal of adrenocorticotrophic hormone (ACTH) (Kerr et al., 1972). Other stimuli include hypoxia, radiation, free radicals and high glucose (Allen et al., 2003; Elmore, 2007).

When intrinsic apoptosis is initiated, mitochondrial outer membrane (MOM) pores are formed releasing the pro-apoptotic protein cytochrome c from the mitochondria to the cytosol, which can bind and activate procaspase-9 and Apaf-1 to form an apoptosome. The apoptosome activates initiator caspase 9. It was found that Bcl-2 family members are responsible for regulating cytochrome c release (Hengartner, 2000).

The first Bcl-2 family member was found in B-cell lymphoma and was therefore named Bcl-2. More Bcl-2 family members with various functions were discovered thereafter. The Bcl-2 family can be divided into three groups: anti-apoptotic proteins, which have four conserved Bcl-2 homology domains (BH-1-BH-4) and examples of this group are Bcl-xL and Bcl-2; pro-apoptotic proteins, whose BH-4 domain is absent compared to anti-apoptotic proteins; BH3-only proteins, which only share sequence homology with the BH-3 domain and they include Bid and Bad (Gross, McDonnell, & Korsmeyer, 1999).

The mechanisms of Bcl-2 family regulated cytochrome c release are:

1. Pore formation by pro-apoptotic proteins

Pro-apoptotic proteins Bax and Bak can permeabilize the mitochondrial outer membrane forming mitochondrial permeability transition (MPT) pores and inducing cytochrome c release (Dewson & Kluck, 2009). BH3-only proteins are divided into activators and sensitizers. Activator proteins (such as tBid and Bim) can bind directly to pro-apoptotic proteins and anti-apoptotic proteins while sensitizer BH-3 proteins (such as Bad, Bik and Noxa) bind only to anti-apoptotic protein. tBID or BIM can directly induce oligomerization of BAX and BAK and cytochrome c release. BIK and Bad can bind to anti-apoptotic proteins and abolish their protective function (Siddiqui, Ahad, & Ahsan, 2015).

2. VDAC-involved cytochrome c release

Voltage-dependent anion channel (VDAC) is located in the MOM and facilitates substance trafficking between the mitochondria and the cytosol. Shimizu et al. found at first that Bax and Bak can bind to and open VDAC allowing cytochrome c release while Bcl-xL can prevent this (Shimizu, Narita, & Tsujimoto, 1999). Later they showed that Bax can form large pores with VDAC on mitochondria causing cytochrome c release (Shimizu, Ide, Yanagida, & Tsujimoto, 2000).

1.4.2.2 Extrinsic pathway

The extrinsic pathway is characterized by apoptosis initiated by death factors binding to their receptors. Well studied death factors are Fas ligand (FasL) and tumor necrosis factor (TNF) and they both belong to TNF family sharing similar extracellular and cytoplasmic domains. Upon binding of FasL to its receptor Fas, the complex induces Fas trimerization and another binding with the adapter protein FADD/MORT1. FADD/MORT1 associates with and activates the initiator caspase 8. For TNF and TNF receptor 1 (TNFR1), the binding trimerizes TNFR1 and recruits TRADD and FADD/MORT1 which activates caspase 8 (Nagata, 1997).

1.4.2.3 Perforin/granzyme pathway

Cytotoxic T cells can kill tumor cells and cells infected with virus mainly through another apoptotic pathway, the perforin/granzyme pathway. Upon activation by cytotoxic T cells, perforin forms pores on the plasma membrane, releasing granzyme A and granzyme B. Granzyme B can activate initiator caspase 10 or directly activate caspase 3 to cause apoptosis of the target cell. Granzyme A incudes apoptosis in a caspase independent way by activating DNA nicking and DNA degradation (Elmore, 2007).

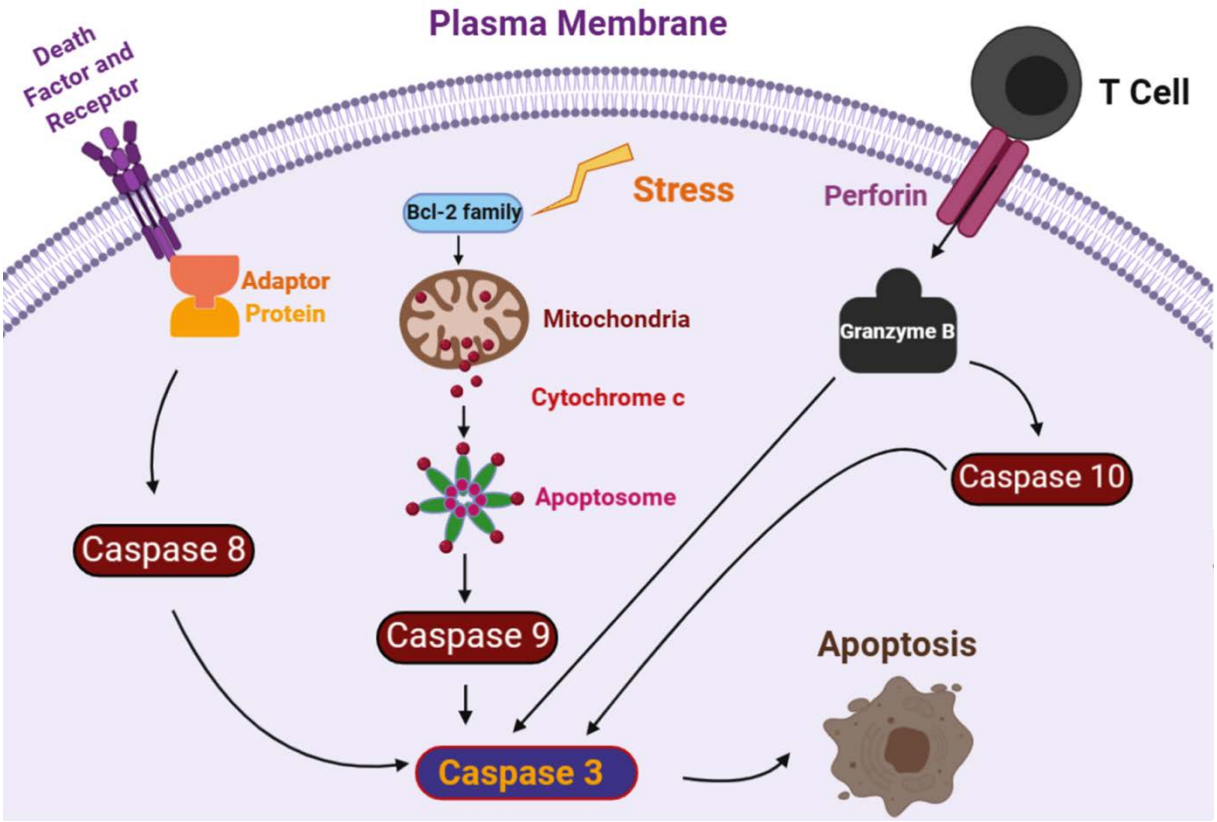

Figure 4: The three apoptotic pathways. The intrinsic pathway starts with intracellular stress which regulates Bcl-2 family proteins. The Bcl-2 family proteins cause MOM pores, releasing cytochrome c. Cytochrome c form apoptosome together with procaspase 9 and Apaf-1, activating caspase 9. The extrinsic pathway is initiated by death factor binding to its receptor, which induces the binding to the adaptor protein. The caspase 8 is thus activated. The perforin-granzyme pathway is activated by cytotoxic T cells. The perforin forms pores on the plasma membrane, releasing gramzyme B. Caspase 8, 9, 10 can all activate caspase 3 and induce apoptosis. The figure is designed using Biorender online tool: https://biorender.com/.

1.4.3 Autophagic cell death

Autophagy is an intracellular degradation process. During autophagy, membranes engulfing molecules or whole organelles are formed in the cytoplasm, i.e. autophagosomes. Lysosomes can fuse to autophagosomes and break them down, allowing their contents to be reused by the body. Autophagy is also called type II or autophagic cell death (Tait et al., 2014).

However, autophagy can also protect cells. Under stressful conditions cells that have ongoing autophagy have better survival rate than cells that do not, i.e. autophagy serves as a self-protecting mechanism for cells under stress. There is some interplay between apoptosis and autophagy: caspase can cleave proteins that initiate autophagy and autophagy can prevent apoptosis by removing damaged mitochondria and other cell material. It has been shown that when apoptosis is blocked, unstoppable autophagy can finally kill cells (Lockshin, 2016).

1.4.4 Necrosis

Necrosis is morphologically characterized by cytoplasmic swelling (oncosis), swelling of cytoplasmic organelles, rupture of the plasma membrane and changes in the nucleus such as pyknosis and karyolysis (Kroemer et al., 2009; Majno & Joris, 1995). When first described necrosis was perceived as accidental and uncontrolled cell death, however it has now been found that necrosis can be programmed and regulated. Programmed necrosis is called necroptosis (Kroemer et al., 2009).

There are several signaling pathways that can initiate necroptosis: TNF can induce necroptosis in certain cell types; death receptor ligands, for example, Fas can initiate necroptosis when caspase activity is inhibited; Toll-like receptors, such as TLR3 and TLR4, also induce necroptosis when caspase is inhibited (Kroemer et al., 2009; Tait et al., 2014). Receptor-interacting serine/threonine-protein kinase 1 (RIPK1) and RIPK3 play a central role in necroptosis signaling. Activation of death receptors or Toll-like receptors initiates RIPK1 and RIPK3 interaction, leading to activation of RIPK3. Once RIPK3 is activated, the downstream mixed-lineage kinase domain-like (MLKL) is phosphorylated and form oligomers which translocate to the plasma membrane and induce plasma membrane lysis (Tait et al., 2014).

1.4.5 Other types of programmed cell death

Pyroptosis is caspase-dependent programmed cell death which has different features from apoptosis. Pyroptosis is dependent on caspase 1 or caspase 11, however, these caspases will not activate executioner caspases. There is rapid plasma membrane lysis while no mitochondrial permeabilisation is found during pyroptosis. Pyroptosis is also inflammatory because caspase 1 is a pro-inflammatory caspase and plasma membrane lysis can release the intracellular contents. In contrast, apoptosis is non-inflammatory due to the intact membranes of the apoptotic bodies (Tait et al., 2014).

Paraptosis is a form of programmed cell death initiated by insulin-like growth factor receptor I and pyronecrosis is the necrotic death of macrophages infected with certain bacteria with Nalp3 and ASC being involved (Kroemer et al., 2009).

1.5 Na, K-ATPase

1.5.1 Discovery, structure and physiology

The Na, K-ATPase, also called sodium pump, belongs to the family of P-type ATPases and was discovered by Skou in 1957. Na, K-ATPase is a plasma membrane protein that transports three Na^+ ions out and two K^+ ions into the cell at the cost of one ATP creating the ion gradients across plasma membrane (Morth et al., 2007).

The sodium and potassium gradients created by Na, K-ATPase are utilized for various physiological activities. For example: (1) in the renal tubules, high expression level of Na, K-ATPase is responsible for filtration and reabsorption to maintain electrolytes and pH in the body; (2) sperm cells express the α4 isoform of Na, K-ATPase, and ion regulation and membrane potential are important for the mobility of sperm cells and fertilization; (3) astrocytes use sodium gradient for neurotransmitter reuptake and action potentials in neurons are also dependent on Na, K-ATPase activity (M. V. Clausen, Hilbers, & Poulsen, 2017).

The Na, K-ATPase consists of three protein subunits: α, β and FXYD. The α subunit has ten transmembrane (TM) helix and three cytoplasmic domains, the actuator (A), phosphorylation (P) and nucleotide-binding (N) domains; the β subunit has one TM helix and a large extracellular terminus; the FXYD subunit has only one TM part. Among the different components of Na, K-ATPase, the α subunit is the functional unit and responsible for binding of Na^+, K^+ and ATP and the transport of ions. The β unit is part of the functional core of Na, K-ATPase and helps α subunit trafficking. FXYD is not an essential component of Na, K-ATPase, and it has more isoform variation and can regulate the pump activity in a tissue-specific manner (M. V. Clausen et al., 2017; Morth et al., 2007).

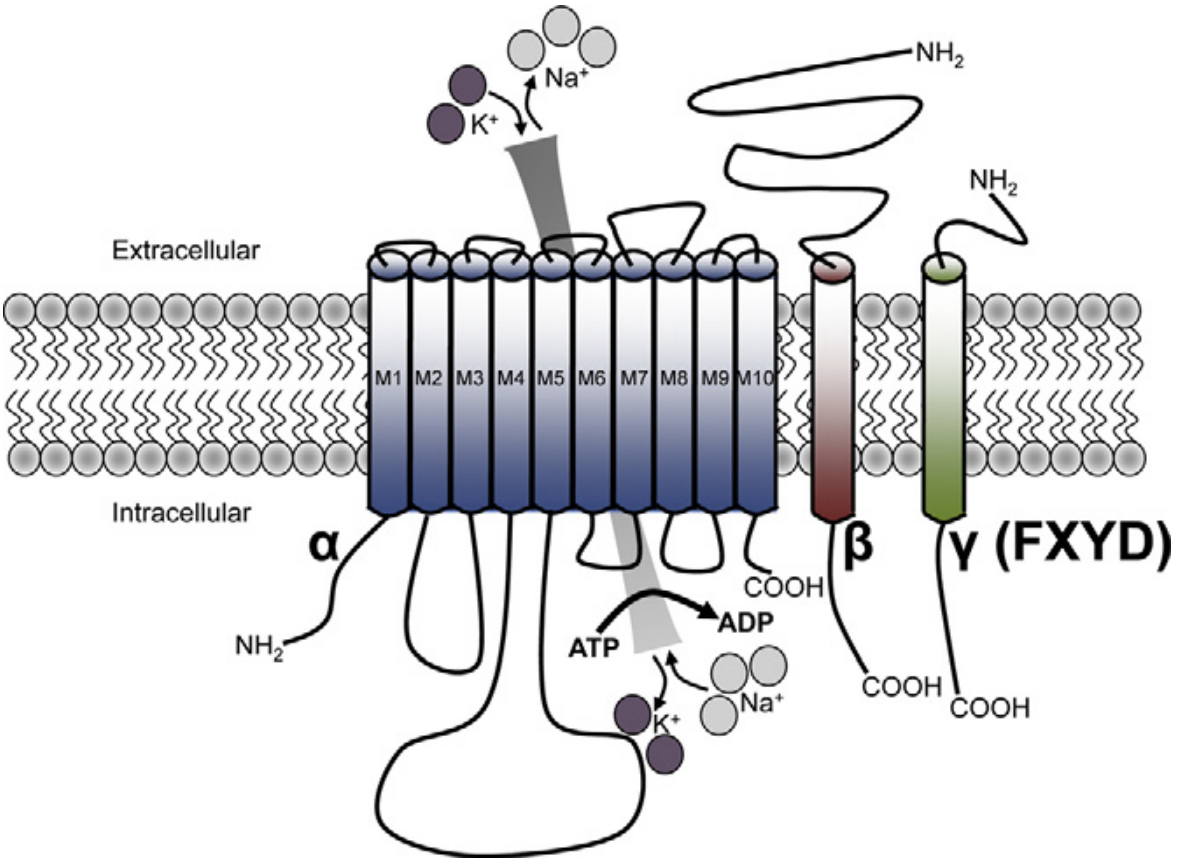

Figure 5: Schematic of Na, K-ATPase structure. The α subunit has ten TM helix named as M1-M10. The cytoplasmic A domain is formed between M2-M3, while the cytoplasmic P and N domains are formed between M4-M5. Both the β subunit and the γ subunit (the FXYD2 subunit) have one TM helix. The figure is reprinted from Bottger et. al (Bottger, Doganli, & Lykke-Hartmann, 2012).

1.5.2 Isoforms and function

There are four isoforms of the α subunit, three β isoforms and seven FXYD isoforms. The most common expressed type of Na, K-ATPase is composed of one α1 and one β1 subunit. Studies have showed that any combination between an α subunit and a β subunit can form a functional pump. In the nephron, the α1β1 type Na, K-ATPase is found (M. V. Clausen et al., 2017).

1.5.2.1 Alpha subunits

The α1, α2 and α3 isoforms are well conserved as they are approximately 87% identical to each other and 78% identical to the α4 in the same species. Among different species, each type of α isoform is also well conserved (M. J. Clausen, Nissen, & Poulsen, 2011; Shamraj & Lingrel, 1994). The variation between different isoforms depends on the surface of the N-domain since other domains are well conserved. Different isoforms have different affinities to

ions and different kinetic properties, for example, α3 has low sodium affinity while α1 has high potassium affinity (Blanco, 2005).

Alpha 1

The α1 isoform is expressed almost in all tissues and cells. In the hearts, all animals express α1 isoform, in some cases together with α2 and/or α3. The sodium and potassium gradients created by Na, K-ATPase are essential for cardiac rhythm and myocardial contraction (M. V. Clausen et al., 2017). During heart failure, there is a decrease in transcription or expression of these three isoforms (Borlak & Thum, 2003; Schwinger et al., 1999).

The α1 isoform is expressed in the renal epithelial cells where Na, K-ATPase are distributed in the basolateral membrane of the polarized epithelial cells (Caplan, Anderson, Palade, & Jamieson, 1986). With this organization, sodium is preserved as Na^+ ions are absorbed from tubule lumens and transported back to the plasma. However, in some tissues or physiological cavities where low level of potassium or high sodium is needed, the Na, K-ATPase are distributed in the apical membrane to transport the K^+ ions back. Examples of these two situations are in choroid plexus and eye respectively (M. V. Clausen et al., 2017).

Alpha 2

The α2 subunit is the dominant form in cardiac and skeletal muscles, as wells as in astrocytes and glia cells. This form is more sensitive to voltage and the α2β2 Na, K-ATPase has extremely low affinity but high capacity for potassium making it efficient at clearing potassium after neuronal activity, as seen in glia cells and astrocytes (M. V. Clausen et al., 2017). The α2 subunit is also close to the Na^+/Ca^{2+}-exchanger in contractile tissue indicating its role in calcium and contractibility regulation (Juhaszova & Blaustein, 1997).

Alpha 3

This isoform is predominantly expressed in brain, especially neuronal projections (Bottger et al., 2011). α3 has a lower sodium affinity than other isoforms and α3 Na, K-ATPase is responsible for clearing high sodium concentrations in the dendrites and spines after neuronal activity (Azarias et al., 2013; M. V. Clausen et al., 2017). Neurodegenerative diseases can be the results of some protein/peptide interaction with α3 subunits. Amyloid-beta, alpha-synuclein and misfolded SOD1 protein can all interact with α3 causing Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis (ALS) respectively (Ohnishi et al., 2015; Ruegsegger et al., 2016; Shrivastava et al., 2015).

Alpha 4

This isoform is less conserved than the other three isoforms and α4 isoform is considered to be sperm specific. Sperm cells express both α1 and α4 isoform, but the α4 isoform is responsible for sperm motility and fertility. Compared to α1, α4 is less sensitive to voltage, temperature and extracellular sodium (M. V. Clausen et al., 2017).

1.5.2.2 Beta subunits

The β subunit of Na, K-ATPase has three isoforms and the structure of them is: a small intracellular N-terminus (30 amino acids), a larger (around 240 amino acids) C-terminus and a TM helix. Besides its involvement in α subunit trafficking, the post-translational modifications of the β subunit can regulate Na, K-ATPase activity (M. V. Clausen et al., 2017). The β1 subunit is the major form in the heart and malfunction of β1 results in heart enlargement and reduced contractility (Barwe et al., 2009). Mice with knocked-out β2 may die due to reduced Na, K-ATPase activity and neural degeneration in their brains (Magyar et al., 1994).

1.5.2.3 FXYDs

FXYD subunits share PFxYD motif in the N-terminus of the TM helix. There are seven isoforms of FXYD in mammals that can regulate Na, K-ATPase activity. FXYD1 is expressed mainly in the brain, heart and skeletal muscle. This isoform inhibits Na, K-ATPase activity (M. V. Clausen et al., 2017). FXYD2 is also called γ subunit and was the first discovered FXYD. FXYD2 is present in kidney. Besides its inhibitory function on Na, K-ATPase, FXYD2 affects mouse reproductivity when the gene is knocked-out (M. V. Clausen et al., 2017). The functions of FXYD3 and FXYD5 are unclear, but there is overexpression of these isoforms in cancer cells. FXYD4 exists in kidney collecting duct and can increase Na, K-ATPase activity. FXYD7 is brain specific and can decrease K^+ affinity (M. V. Clausen et al., 2017). FXYD6 can increase Na, K-ATPase activity and increased FXYD6 has been found in hepatocellular carcinoma and mental disorders (Gao et al., 2014).

1.5.3 Na, K-ATPase in diseases

There are no genetic diseases related to mutations in the β subunit or FXYD reported, while several mutations in α subunits have been shown to be responsible for a variety of diseases.

Mutation of the α1 subunit gene, ATP1A1 is related to aldosterone overproduction and hypertension. Various mutation sites at α2-encoded ATP1A2 are responsible for Familial Hemiplegic Migraine (FHM), an inherited migraine with weakness and aura in one side of the

body. Increased level of extracellular potassium and glutamine maybe the mechanism behind FHM caused by ATP1A2 mutation. Mutation in ATP1A3 has been reported to cause several neurological syndromes: Rapid-onset Dystonia Parkinsonism (RDP), CAPOS (Cerebellar ataxia, areflexia, pes cavus, optic atrophy, sensorineural hearing loss) and Alternating Hemiplegia of Childhood (AHC) (M. V. Clausen et al., 2017).

1.6 Ouabain

1.6.1 Ouabain in nature and endogenous ouabain

Ouabain, a cardiotonic steroid (CTS) hormone, was initially identified and named by Arnaud in 1888. The natural form of ouabain is found in plants: from the bark of a Somali tree called “ouabaio” and the seeds of Strophanthus kombe and Strophanthus gratus (Blaustein, 2018). In 1991, Hamlyn and his colleagues identified and characterized ouabain-like compound (OLC) from human plasma samples and later named it as endogenous ouabain (EO). EO and natural ouabain are identical compounds analyzed by mass spectrometry (Hamlyn et al., 1991). The structure of ouabain is shown below: it consists of the steroid nucleus, the binding lactone ring and rhamnose.

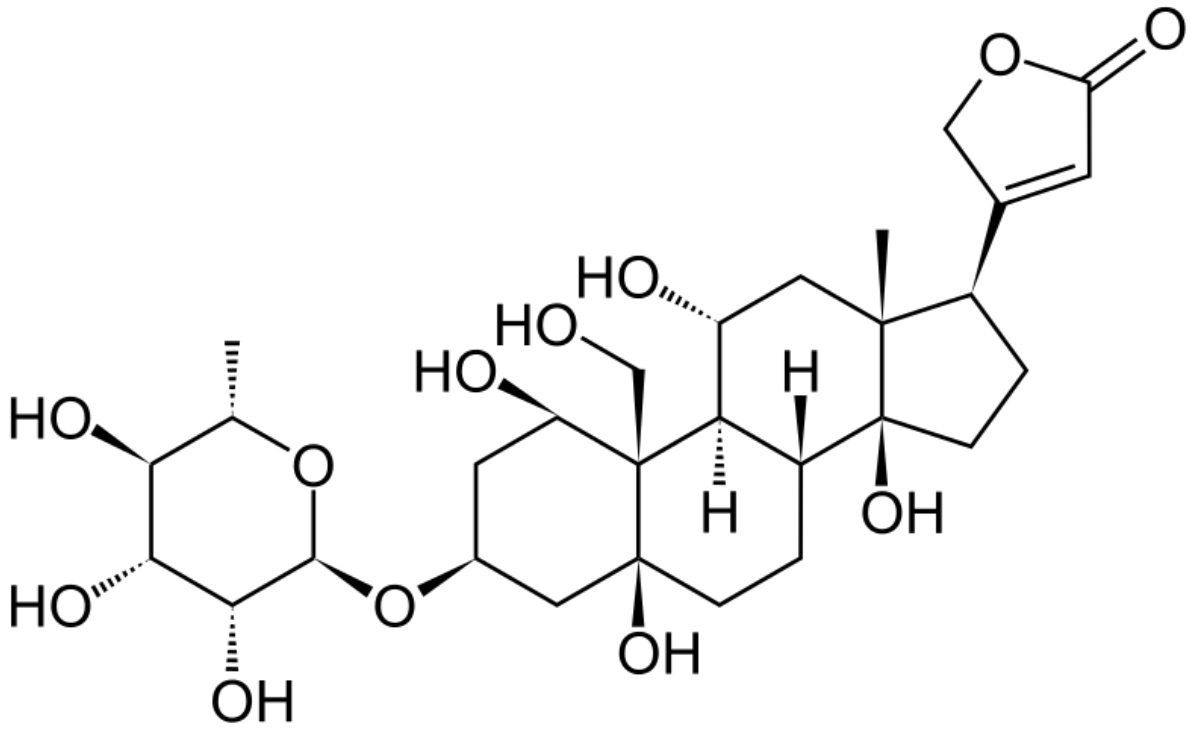

Figure 6: Structure of ouabain

Marinobufagenin and telocinobufagin are two examples of other endogenous CTS, both of which belong to bufadienolide. The former is found in amphibian (Blaustein, 2018) and both of the compounds are reported to increase in renal failure patients (Komiyama et al., 2005). However, ouabain is in dominant presence among all endogenous CTS (Blaustein, 2018).

In spite of the fact that EO has been found in human and rodent blood sample, bovine adrenal and even human placenta samples (Blaustein, 2018), some scientists doubt about the very existence of EO. In his review paper, Nicholls and his colleagues argued that the mass spectrometry (MS) method used to discover EO is outdated and state-of-art MS equipment should be used to confirm the existence of EO. Besides, one study showed that when a selective ouabain inhibitor rostafuroxin was applied to patients with systolic hypertension, there was no change of blood EO levels or blood pressure (Lewis et al., 2014). Baecher et al. have applied an ultra-sensitive UPLC-MS/MS method that can detect ouabain at concentration as low as 1.7 pmol/L, and yet they did not detect any EO in the human plasma (Baecher, Kroiss, Fassnacht, & Vogeser, 2014). Blaustein argued that an ion current corresponding to isomers of ouabain actually appeared in their MS-MS ion spectra and the authors have neglected it (Blaustein, 2018). There is still ongoing debate about the presence of EO and both sides seem to have a firm stand.

1.6.2 Ouabain as a drug and pump inhibitor

Digitalis and its CTS homologs have been used to treat congestive heart failure for more than 200 years. The mechanism behind this is that CTS can inhibit Na, K-ATPase specifically and increase intracellular sodium levels, which increases Na^+/Ca^{2+} exchanger activity. Increased intracellular Ca^{2+} will have inotropic effect on cardiac muscle (Schoner, 2002). Many research groups have used high concentration of ouabain as a tool to block Na, K-ATPase pump activity in experimental models.

Ouabain was reported to induce hypertension in rats and this effect was once believed to associate with its sodium pump inhibition. However, other CTSs which also inhibit the pump activity do not increase blood pressure: digoxin has been used for over two centuries without any report of a hypertensive effect. On the contrary, it has been reported that other CTS can counteract ouabain's hypertensinogenic effect. Lichtstein et al. suggested the ouabain-digoxin antagonism as low-dose ouabain also rescues cardiomyocytes from digoxin-induced cardiotoxicity (Nesher et al., 2010). Further evidence to show that inhibition of sodium pump activity is not responsible for ouabain-induced hypertension is that a small modification of ouabain lactone ring can decrease dramatically its pump inhibitory effect but enhance its hypertensinogenic effect (Manunta, Hamilton, & Hamlyn, 2001). It is now suggested that ouabain signaling mechanism is responsible for hypertension and Na, K-ATPase subunit α2 is the target of ouabain, as mice with ouabain-resistant α2 genotype are resistant to ouabain-induced hypertension (Blaustein, 2018).

1.6.3 Endogenous ouabain and physiological roles

EO was reported to have an important physiological role since its discovery. Physiological levels of EO is required for normal organ function and embryonic development, while either elevated or decreased level of EO can be detrimental to mammals.

Cardiovascular system

Hypertensinogenic effect of EO maybe the reason why EO level was elevated in heart failure patients; EO has negative correlation to cardiac index (Gottlieb et al., 1992) but positive correlation to cardiac hypertrophy (Stella et al., 2008). Elevated EO is also responsible for worse clinical outcome (Simonini et al., 2015).

Neurological effect

When rats were injected with low-dose ouabain, they exhibited mania-like behavior (El-Mallakh et al., 2003). Inhibition of EO with specific ouabain antibody can attenuate symptoms in animal models of mania and depression respectively (Goldstein et al., 2012; Hodes et al., 2016). It was suggested that EO could not pass through blood-brain-barrier, thus the reason why EO affects learning and behavior need to be more studied. Some group brought up the theory that EO can originate from the brain (Blaustein, 2018).

Stress hormone

EO acts as a stress hormone as rapid elevated levels of EO were seen after cardiac surgery, salt excess/depletion in diets and intense physical activity (Blaustein, 2018).

Growth factor

EO is also a growth factor. The malnutrition of the pregnant mouse will impair kidney development of embryos, and ouabain can restore the renal development in malnutrition during pregnancy (Li et al., 2010). Using specific ouabain antibodies will affect embryo development with reduced kidney and liver volume, and in pregnant women, low level of circulating ouabain is an indicator for slow fetal development (Dvela-Levitt et al., 2015).

1.6.4 Ouabain and Na, K-ATPase signaling pathway

As discussed in section 1.6.2, ouabain can induce hypertension through Na, K-ATPase signaling which indicates that Na, K-ATPase is not just a pump that creates gradient across plasma membrane. In fact, ouabain can induce a cascade of signaling through Na, K-ATPase.

1.6.4.1 Ouabain/Na, K-ATPase/IP3R/NF-κB pathway

It has been shown by several groups that ouabain can stimulate cell proliferation in different cell types: cardiomyocyte, renal cell, vascular smooth muscle cell and astrocyte et al (Aydemir-Koksoy, Abramowitz, & Allen, 2001; Li, Zelenin, Aperia, & Aizman, 2006; Murata et al., 1996; Xie & Askari, 2002). Our group showed ouabain stimulates cell proliferation of PTC and COS7 cells. Ouabain also protects cells from serum starvation-induced apoptosis through ouabain/Na, K-ATPase interaction which activates 1,4,5-trisphosphate receptor (IP3R), triggering slow calcium oscillations and activating NF-κB (Aizman, Uhlen, Lal, Brismar, & Aperia, 2001; Li et al., 2006). We showed later that the ouabain/Na, K-ATPase/IP3R/NF-κB pathway increases expression of Bcl-xL and rescues cells from apoptosis induced by shiga-toxin, high glucose and high level of albumin (Burlaka et al., 2013; Burlaka et al., 2016; Nilsson et al., 2019).

1.6.4.2 Ouabain/ Na, K-ATPase/Src pathways

Binding of ouabain to Na, K-ATPase can active Src protein kinases in different cell types, and this was shown by Xie and his colleagues (Haas, Askari, & Xie, 2000; Haas, Wang, Tian, & Xie, 2002). Immunoprecipitation result showed there was direct interaction between Na, K-ATPase and Src and ouabain can increase this physical interaction in a dose- and time-dependent manner (Xie & Cai, 2003). It was also discovered by Xie et al. that W423, L424 and R427 in the N-terminus of α1 Na, K-ATPase is responsible for Na, K-ATPase /Src binding. Src kinase activation can induce tyrosine phosphorylation of numerous proteins such as epidermal growth factor receptor (EGFR). EGFR/Ras/Raf/MEK/MAPK (also called ERK 1/2), EGFR/Ras/ROS and PLC/PKC are three down-stream signaling pathways of ouabain/ Na, K-ATPase/Src (Cui & Xie, 2017).

These pathways were discovered in cardiomyocytes whose proliferation was stimulated by ouabain, indicating that they are rescuing signaling. Fontana et al. showed that the use of the Src inhibitor can abolish calcium activities induced by ouabain, indicating that Src played a major role in ouabain-induced rescue signaling (Fontana, Burlaka, Khodus, Brismar, & Aperia, 2013). MAPK (mitogen-activated protein kinase) activation by ouabain is confirmed in study I of the present thesis when we treated COS7 cell with ouabain. Interestingly, we found in study II that ouabain had an antioxidant effect as it can reduce ROS production in PTC and mesangial cells which is different from previous study where mitochondrial origin ROS production was increased by ouabain (Haas et al., 2002; Liu et al., 2000).

Na, K-ATPase/Src pathways is mediated on α1 unit of Na, K-ATPase as other isoforms of α unit do not interact with Src. However, it was reported that in α3 isoform expressing cells the same signaling pathway can be activated independent of Src (Cui & Xie, 2017).

1.6.5 Other signaling pathways

In addition to the pathways described above, ouabain can also act on PI3K/PDK/Akt in cardiac myocytes, however, this pathway is directly activated by ouabain /Na, K-ATPase and independent of Src activation (Wu et al., 2013).

Furthermore, ouabain /Na, K-ATPase signaling pathway can regulate cell tight junction and gap junction (Larre et al., 2010; Larre, Ponce, Franco, & Cereijido, 2014; Ponce et al., 2014). It has been shown that ouabain can decrease tumor cell migration and tumor cell growth. In these studies, ouabain was shown to either activate ERK 1/2 or inhibit Akt activity, both are detrimental to cancer cells (Kometiani, Liu, & Askari, 2005; Pongrakhananon, Chunhacha, & Chanvorachote, 2013). The rescuing effect on normal cells and detrimental outcome on tumor cells of ouabain makes ouabain a potential drug in the future as it selectively affects cells based on their phenotypes.

2 Aims

2.1 Study I

To investigate the universal signaling pathways generated by ouabain/ Na, K-ATPase. To study this, we performed proteomics and phosphoproteomics experiments on COS7 cells treated with ouabain.

2.2 Study II

To study which renal cell type is most vulnerable to hyperglycemia and the cellular mechanism behind this using a primary renal cell culture model treated with high glucose. To check if ouabain can protect renal cells from high glucose induced apoptosis.

2.3 Study III

To reveal the molecular mechanism behind high glucose induced apoptosis and anti-apoptotic signaling of ouabain.

3 Material and Methods

3.1 Cell line and primary cell culture

In my thesis work, I have used both a cell line and primary cell cultures. The cell line, COS7 is derived from monkey kidney tissues, and it is commonly used to study biological questions. COS7 cells are fibroblast-like and are arranged in a monolayer when cultured on a flat surface. They are easily passaged and proliferate rapidly, and more importantly, we have verified that they have a preserved phenotype up to 15 passages. COS7 cells can be easily transfected which makes them suitable for plasmid or siRNA transfection to study gene function and label protein intracellularly. Primary cells are normally prepared directly from an organ or tissue, and therefore considered more representative of the biological system than cell lines. Primary cells, however, can be passaged for only a few generations because of a change of phenotype. Primary PTC, podocyte and mesangial cell cultures were used in our study to observe their response to glucose and ouabain treatment.

In study I, COS7 cells were used for ouabain treatment and then proteomics analysis, live cell calcium imaging with GCaMP6 transfection, sodium imaging with a sensitive sodium dye and Western blot analysis. Proteomics analysis needs plenty of biological materials and COS7 cells are easy to produce, in addition, COS7 cells are easily transfected with GCaMP6. To confirm that ouabain induced the same signaling pathways in primary cells as in COS7 cells, we also used primary PTC culture. Performed experiments on primary PTCs include gene down-regulation, high-glucose or serum-deprivation-induced apoptosis analysis.

In study II, the majority of the experiments were performed on rat primary PTC, podocytes and mesangial cells. Our lab has established protocols for PTC, podocyte and mesangial cell culture. Since these cells are derived from kidneys of the same rat strain, it is easy to compare certain protein and gene expression levels among them, as well as different responses to high glucose treatment. To compare distinct glucose response between primary podocytes and podocyte cell lines, we used an immortalized podocyte cell line.

In study III, we focused on primary PTC to study the molecular mechanism behind high-glucose-induced apoptosis and how ouabain protected from apoptosis. Live cell imaging, apoptosis detection as well as immunocytochemistry were performed on PTC.

3.2 Kidney cortex tissue

The yield from our primary PTC preparation is rather low considering the large PTC numbers in the kidney cortex. To harvest large amount of protein for immunoprecipitation experiment, we set up a protocol to use live kidney cortex tissue directly from the kidney instead of

preparing primary PTC culture. The cortex tissue was treated for one hour in vitro after dissection and then homogenized for protein extraction.

3.3 Patient material

In study II, we examined apoptosis in kidney biopsies from both DN patients and kidney donors. The biopsies were taken for diagnostic purpose or for health assessment of the grafts. Renal tubules with apoptosis were significantly more present in DN patients than in healthy donors, and this was consistent with in vitro observation where high glucose induced apoptosis in primary PTC. These finding imply the great value of our study on primary cultures.

3.4 Apoptosis detection

Since apoptosis is an orchestrated cascade of events, there are different assays for detecting each particular event in the apoptotic process, e.g. Annexin V, caspase activity assays and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). However, each method has its advantages and disadvantages to detect apoptosis, besides, apoptosis and necrosis share some features such as DNA fragmentation making it hard to distinguish between these two pathways. It is therefore suggested to apply at least two apoptosis assays for confirmation of apoptosis (Elmore, 2007). In this study, we have used the TUNEL assay, immunostaining of Bcl-2 family proteins as well as mitochondria membrane potential (Ψm) and ROS measurement to study apoptosis. By applying TUNEL staining, we were able to sensitively detected single-cell level apoptosis. While using immunostaining, Ψm and ROS measurement, we can study the apoptotic process and the underlining mechanism of high glucose induced apoptosis.

3.4.1 TUNEL assay

The TUNEL assay was introduced in 1992 as Gavrieli and his colleagues claimed that TUNEL was a much better method than DNA laddering: in situ visualization of apoptosis at a single-cell level while maintaining the original structure of the tissue. The apoptosis seen by means of TUNEL staining start at the nuclear periphery and apoptosis appears in clusters in tissues. The time window for apoptosis detection is about 1 to 3 hours (Gavrieli, Sherman, & Ben-Sasson, 1992). In TUNEL labelling, terminal transferase is applied to enable nucleotides (dUTP) binding to the 3’ end of DNA fragments. The dUTP can then be labelled with different probes and detected by light microscopy or flow cytometry. One commonly used

probe is digoxigenin and the labelled dUTP can be detected with anti-digoxigenin antibodies conjugated with a fluorescent dye. TUNEL staining is sensitive and can detect a single cell under apoptosis. (Watanabe et al., 2002).

3.4.2 Mitochondrial parameters

Mitochondria are the platform of the intrinsic apoptotic pathway. Ψm, MPT, ROS and calcium fluxes can be used as mitochondrial health parameters. In addition, immunohistochemistry and Western Blot analysis of cytochrome c, as well as Bcl-2 family proteins can be used as mitochondrial assays (Watanabe et al., 2002).

3.5 Light microscopy

Light microscopy is widely used in cell biology for cell imaging and it can be divided into bright-field microscopy and fluorescence microscopy. In bright-field microscopy, light passes through the sample as the light source and detection objective are located on the opposite sides. This makes the cells difficult to observe when they do not absorb enough light. In fluorescence microscopy, fluorescent dyes are used to label molecules of interest. Fluorescent dyes are excited with one wavelength of light and emit another. This means that specific proteins with low abundance can be detected (Thorn, 2016). In my thesis, I used fluorescence microscopy: confocal microscopy and super resolution microscopy (both STED and STORM).

3.5.1 Confocal microscopy

The basic principle behind confocal microscopy is the use of a pinhole to generate a focused spot of illumination. In wide-field microscopy, there is lower signal-to-noise ratio in the image compared to confocal microscopy because the image is composed of in-focus light as well as out-of-focus light coming from outside the focal plane. The confocal microscope has a pinhole which can remove out-of-focus light, therefore achieve better quality image than wide-field microscopy (St. Croix, Shand, & Watkins, 2005). Confocal microscopy has been widely used in live cell and immunocytochemistry imaging. In our study, we used confocal microscopy for live cell imaging, as well as fixed cell imaging to study immune-stained Bax and Bcl-xL accumulation on mitochondria in study II.

3.5.2 Super resolution microscopy

When light waves pass through an aperture smaller than half of the wavelength of the light or, are focused on a small spot, there is a “spreading out” phenomenon called diffraction (Huang, Babcock, & Zhuang, 2010). Because of the light diffraction, the resolution of confocal microscopy is limited to around 200 nm. Fine cellular structures which are smaller than 200 nm can therefore not be resolved. Several super resolution methodologies have been developed to achieve higher resolution imaging of biological samples. These new microscopy techniques include stimulated emission depletion microscopy (STED), photoactivated localization microscopy (PALM), structured-illumination microscopy (SIM), fluorescence photoactivation localization microscopy (FPALM), and stochastic optical reconstruction microscopy (STORM). In STED, a donut-shaped laser beam is applied overlapping the excitation spot in the focal region, which can deplete any fluorescence outside the center of the excitation spot to reach a resolution of 20 nm (Leung & Chou, 2011). STORM is based on high-accuracy localization of photoswitchable fluorophores; during one imaging cycle, a fraction of the fluorophores are randomly turned on, and after a series of cycles, the overall image is reconstructed with a possible resolution of 20 nm (Rust, Bates, & Zhuang, 2006). In study III, we performed STED and STORM to study possible protein-protein interactions.

3.6 Proteomics

The proteome includes all the proteins encoded by the genome, and the study of the proteome is called proteomics. The study on the proteome-wide phosphorylation events is called phosphoproteomics (Tyers & Mann, 2003). The dynamic change of proteome is more justifiable to represent the gene expression levels than genome. Proteomics and phosphoproteomics can be used for protein profiling, protein modification and protein-protein interaction analysis, drug development, as well as to discover new biomarkers in disease (Aebersold & Mann, 2003; Hanash, 2003).

HiRIEF LC-MS

Mass spectrometry (MS)-based proteomics has become a popular tool for protein sample analysis. In MS-based proteomics, the protein samples are first digested generating peptide fragments. The electrospray ionization technique is then applied to ionize the peptide fragments. Each peptide will have a unique mass-to-charge ratio (m/z) value and the value will be measured by a mass analyzer and registered by a detector. Integrated liquid-chromatography- MS (LC-MS) technique combines the physical separation property of LC and mass analysis property of MS and is recommended for complex peptide mixture analysis (Aebersold & Mann, 2003). In study I, we applied high-resolution isoelectric focusing (HiRIEF) LC-MS. HiRIEF LC-MS applies HiRIEF gel strips to prefractionate peptide samples. The peptides will be separated depending on their different isoelectric points and

analyzed by LC-MS later. HiRIEF has been reported to enable deep proteome coverage (Branca et al., 2014).

3.7 Live cell imaging

3.7.1 Calcium imaging

Ca^{2+} generates versatile intracellular signals that regulate a variety of cellular processes that range from cell proliferation to cell death, as well as physiological activities, such as fertilization, development, muscle contraction, etc. Calcium signaling has various patterns, with different amplitude, frequency and duration, which contribute to the versatility of calcium signaling. (Berridge, Lipp, & Bootman, 2000). Calcium imaging facilitates the observation of calcium signaling pattern, as well as its localization in a specific cellular sub-compartment. The development of calcium imaging relies on the improvement of calcium sensors and calcium imaging devices. There are four groups of calcium indicators: bioluminescent protein, chemical calcium indicator, Förster resonance energy transfer (FRET)-based genetically encoded calcium indicator (GECI) and single-fluorophore GECI. Commonly used calcium imaging devices include wide-field microscopy, confocal microscopy and two-photon microscopy (Grienberger & Konnerth, 2012).

Bioluminescent proteins are derived from marine organisms and are among the earliest calcium indicators. Bioluminescent proteins undergo conformational changes upon binding of Ca^{2+} which leads to the oxidation of coelenterazine to coelenteramide. Coelenteramide emits a photon of 470 nm while relaxing to the ground state. However, continuous calcium imaging needs exogenous coelenterazine because coelenterazine has a slow recharging process and coelenteramide can only emit once. Moreover, calcium imaging using bioluminescent proteins has low quantum yield (Grienberger & Konnerth, 2012).

Chemical calcium indicators consist of a fluorophore and a calcium chelator. Upon binding of calcium ions, the molecule undergoes conformational changes resulting in the change in emitted fluorescence. There are a variety of chemical calcium indicators and they can be either membrane-permeable or membrane-impermeable. Chemical calcium indicators have good signal-to-noise ratio and are suitable for both one-photon and two-photon excitation. However, research has shown that commonly used chemical calcium indicators, Rhod-2 acetoxymethyl (AM), Fluo-4 AM, Fura-2 AM and BAPTA AM can suppress Na, K-ATPase activity by 30% to 80% in a set of cells, such as PTC and cardiomyocytes and they may reduce spontaneous calcium signaling. Chemical calcium indicators may also reduce cell viability and alter cell metabolism. (Grienberger & Konnerth, 2012; Smith et al., 2018).

GECIs can be divided into FRET-based GECIs and single-fluorophore GECIs. FRET-based GECI has a donor and an acceptor protein, and binding of calcium will enable energy transfer between the two fluorophores leading to a change of emission pattern of the indicator. Single-fluorophore GECIs have a calcium-binding calmodulin and calmodulin-binding peptide on

each side respectively. Binding of calcium ions will generate conformational change and increase emitted fluorescence (Grienberger & Konnerth, 2012). GECIs have several advantages over bioluminescent proteins and chemical calcium indicators. GECIs are intracellularly stable and allow for long term imaging and can be targeted to a specific organelle. Chemical calcium indicators, however, tend to accumulate in compartments and be extruded from the cells after some time. Importantly, GECIs do not suppress Na, K-ATPase activity or reduce cell viability (Paredes, Etzler, Watts, Zheng, & Lechleiter, 2008; Smith et al., 2018).

In study I, we performed calcium imaging on COS7 cells treated with ouabain. Cells were transfected with the single-fluorophore GECI, GCaMP6, as the calcium sensor imaged with confocal microscopy.

3.7.2 Sodium imaging

Like calcium ions, sodium ions are also reported to act as a second messenger that regulates cellular signaling pathways in both physiological and pathological conditions. The extracellular Na^+ concentration is 10-20 times higher than the intracellular Na^+ concentration in all organisms and this concentration gradient is created by the activity of pumps and ion channels/transporters (Iamshanova, Mariot, Lehen'kyi, & Prevarskaya, 2016). Sodium imaging is challenging because of the relatively small difference of Na^+ concentration across the plasma membrane (for calcium ions, extracellular Ca^{2+} can be 10,000 times higher than intracellular Ca^{2+}). There are several approaches to record Na^+, such as ^{23}Na nuclear magnetic resonance spectroscopy, flow cytometry, patch-clamp technique and fluorescence probes. Among these approaches, fluorescence probes are considered to be both sensitive to sodium change and easy to use. Commercially available Na^+ fluorescence indicators include Na^+-binding benzofuran isophthalate (SBFI), CoraNa™ Green (Corona), and Asante NaTRIUM Green-2 (ANG-2). Among them, Corona has lower sensitivity for small intracellular Na^+ changes compared to SBFI and ANG-2 (Iamshanova et al., 2016). In study I, we used ANG-2 to do intracellular Na^+ recordings to study whether a given concentration of ouabain inhibits Na, K-ATPase pump activity.

3.7.3 Glucose uptake

PTC, podocytes and mesangial cells may express different glucose transporters as previously stated in this thesis. SGLT2 co-transport one glucose molecule and one sodium ion. SGLT1 co-transport one glucose molecule and two sodium ions, while GLUT transporters are independent of sodium ions. We compared sodium-independent glucose uptake in PTC, podocyte and mesangial cell to confirm that PTCs express SGLT2, mesangial cells express SGLT1 and podocyte express GLUTs. 2-[N-(7-Nitrobenz-2-oxa-1, 3-diazol-4-yl) amino]-2-

deoxy-D-glucose (2-NBDG) is a fluorescent D-glucose analog and can be taken up by cells in a competitive manner with D-glucose. When 2-NBDG enters the cell, it is phosphorylated at the C-6 position and turned into a non-fluorescent state. Therefore the fluorescence intensity reflects the equilibrium level of intracellular 2-NBDG. Using 2-NBDG has several advantages over radiotracers like 2-deoxy-D-[^{3}H] glucose and 2-deoxy-D-[^{14}C] glucose: it can detect glucose uptake in single-cell level, is easier to visualize and leaves no radiation hazard (Zou, Wang, & Shen, 2005).

3.7.4 ROS detection

Hyperglycemia plays a major role in diabetic complications and ROS overproduction induced by hyperglycemia can trigger signaling pathways that cause cell death in diabetes (Volpe, Villar-Delfino, Dos Anjos, & Nogueira-Machado, 2018). ROS include free radicals (e.g. O_2^-, ^{1}O2 and •OH) and hydrogen peroxide (H_2O_2) (Eruslanov & Kusmartsev, 2010). In study II and III, we measured cell ROS production to see if cells are under oxidative stress after high glucose treatment. 2', 7'-Dichlorodihydrofluorescein Diacetate (DCFH-DA) is the most wildly used dye for ROS detection. DCFH-DA is sensitive to intracellular ROS changes, and can reveal ROS changes over time. The molecular mechanism behind this method is that DCFH-DA can be cleaved by intracellular esterases to H_2DCF, which can be oxidized by free radicals and then becomes the highly fluorescent DCF. Because DCF is membrane permeable and can diffuse through plasma membrane, we used an improved version of DCFH-DA, di (acetoxymethyl ester) (6-carboxy-DCFH-DA), which has better cellular maintenance for long term imaging as well as higher membrane permeability (Eruslanov & Kusmartsev, 2010).

3.7.5 Mitochondrial membrane potential measurement

The respiratory chain localizes in the MIM and protein complex I, III, IV of the respiratory chain can pump protons out of the mitochondrial matrix creating a Ψm across the MIM (Chen, 1988; DiMauro & Schon, 2003). Ψm is essential for cellular health and viability and loss of Ψm is a predictor of apoptosis (Green & Kroemer, 2004). We measured Ψm on PTC treated with high glucose with or without ouabain in study III. There are many fluorescent probes for Ψm measurement such as DiOC6 (3), rhodamine 123 and TMRM. Here we used the JC-1 dye to distinguish mitochondria with different Ψm: the higher the membrane potential, the higher red/green fluorescence ratio will be. Compared to fluorescent probe DiOC6 (3) and rhodamine 123, JC-1 staining is not affected by plasma membrane potential and can thus generate more reliable result (Salvioli, Ardizzoni, Franceschi, & Cossarizza, 1997; Zorova et al., 2018).

3.8 Immunocytochemistry

Immunocytochemistry is a commonly used method to study protein localization in cells. In immunocytochemistry, a primary antibody is used to identify and bind to the specific epitope of the protein of interest. A second antibody conjugated with a fluorophore is then applied to bind the primary antibody, thus the protein of interest can be visualized. The primary antibody can also be conjugated with a fluorophore in which case the second antibody is not needed. In the present thesis, I used either fluorophore conjugated primary antibodies or normal primary antibodies with secondary antibodies. The fixation method and immunostaining protocol were optimized to fit the antibodies properties. Negative control samples were prepared in each experiment to ensure specificity of the antibody.

3.9 Protein complex immunoprecipitation

Protein complex immunoprecipitation (Co-IP) is a commonly used technique to study protein-protein interactions. In study III, we performed Co-IP to study Bad/Akt interactions. During Co-IP, the antibody is attached to a sedimentable matrix and the matrix is then used to bind specific antigens (proteins) from the cell, tissue lysis, or even protein fractions separated from gel filtration. The technique takes three stages. In stage 1, cells or tissues are lysed, here we used kidney cortex and the tissue was homogenized in lysis buffer with a homogenizer. In stage 2, a specific antibody is attached to a sedimentable matrix, such as protein A- or protein G-agarose beads and covalently coupled to Sepharose. In our study we used magnetic beads which are easy to separate with a magnetic separation rack. Stage 3 is the immunoprecipitation in which the beads are incubated with the tissue lysate. After these three stages, the binding antigens are removed from bead and used for Western blot analysis.

3.10 RNA study

3.10.1 siRNA

Short interfering RNA (siRNA) suppresses target gene expression and is a widely used experimental tool for gene down-regulation. In study II and study III we down-regulated *CaMKK1*, *CaMK2G* and *SLC5A2* (gene for SGLT2). The siRNA primers were commercially available and the down-regulation efficiency was assessed by both real-time qPCR and Western blot to make sure that the target gene was silenced.

3.10.2 Polymerase chain reaction

Polymerase chain reaction (PCR), a process to amplify DNA sequences, has wide application in molecular biology. RT-PCR (reverse transcription PCR) was used in study I to assess gene expression levels of SGLT1 and SGLT2 in different renal cells, and the PCR product was analyzed by electrophoresis on agarose gels. Real-time qPCR was performed in all three studies to compare mRNA levels between control group and siRNA silenced group or control group versus treatment group. Real-time data was analyzed by the comparative C_T method. First of all, as a relative quantification method, comparative C_T method has advantages over absolute quantification methods: easier generation of standard curve and ability to facilitate data analysis when the absolute cycles of PCR are large. Secondly, the comparative C_T method can present the data as fold change to easily compare gene expressions (Schmittgen & Livak, 2008). When we designed PCR primers, we first acquired the coding sequence information from Gene – NCBI. Secondly, we copied the coding sequence information and designed the primers using an online tool, Primer 3 website: http://bioinfo.ut.ee/primer3-0.4.0/. The specificity of primers was checked with Primer – BLAST tool.

4 Results

4.1 Study I

4.1.1 Ouabain treatment induces thousands of phosphorylation events

It was reported previously that low concentration of ouabain can induce slow calcium oscillations on PTC eliciting activation of the transcription factor, NF-κB. These oscillations are independent of ouabain's inhibition of the sodium pump and rely on activities of IP3R (Aizman et al., 2001). Later it was found that treatment of COS7 cells with 100 nM ouabain induced slow calcium oscillation while inhibiting less than 10% of the Na, K-ATPase activity. Na, K-ATPase interacts with the IP3R through the N-terminus of the Na, K-ATPase α-subunit and the N-terminus of the IP3R initiating the Ca^{2+} oscillatory signal to protect cells from apoptosis (Zhang et al., 2006).

In this study we confirmed that 100 nM ouabain induced calcium oscillation in COS7 after 10 to 20 minutes treatment. This concentration of ouabain affected sodium pump activity at a negligible level as intracellular sodium level remained stable during the treatment. Three groups of COS7 cells were included for Standard HiRIEF LC-MS (proteomics) and Phospho HiRIEF LC-MS (phosphoproteomics) analysis: control, 10 minutes ouabain treatment and 20 minutes ouabain treatment. In all samples, proteomics analysis identified 52,599 unique peptides corresponding to 7740 proteins and 7108 genes. Phosphoproteomics analysis identified a total of 15,348 phosphorylation sites with 12,903 on serine residues, 2,323 on threonine residues and 122 on tyrosine residues respectively and the identified phosphorylation sites correspond to 3937 protein-coding genes. We then compared the proteomics and phosphoproteomics profiles between the control and treatment groups. Upon ouabain treatment of 10 mins and 20 mins, 80 and 48 proteins are significantly regulated respectively. However, oubain treatment caused more phosphorylation and dephosphorylation events as 1941 phospho-sites were regulated after 10 mins treatment and 1484 phospho-sites were regulated after 20 mins (Figure 7).

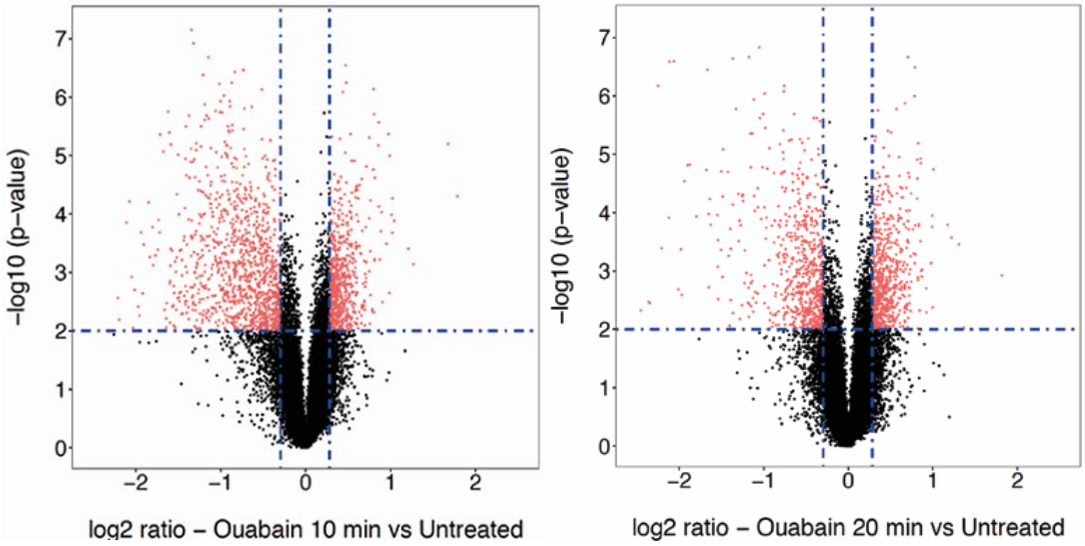

Figure 7: Volcano plots showed significantly regulated phospho-sites represented by red dots and unregulated phosphor-sites were represented as black dots. The left panel shows phospho-sites following 10-minute treatment and the right panel following 20-minute treatment.

Gene ontology analysis showed that among the phosphorylated proteins, the ones that regulate cell junctions and cell proliferation were enriched. This implied that ouabain/ Na, K-ATPase signaling may regulate multiple proteins involved in cell proliferation and cell junctions. Protein kinases can modify proteins by phosphorylating them (Hunter, 1995). Interestingly, protein kinase activity is also regulated by phosphorylation/dephosphorylation process. We found that ouabain treatment regulated phospho-sites on multiple protein kinases including MAPK, epidermal growth factor receptor (EGFR) and Ca^{2+}/calmodulin-dependent protein kinase (CaMK). Dephosphorylation of MAPK by ouabain treatment was further confirmed by Western blot.

4.1.2 New players in oscillatory calcium signal and anti-apoptotic pathway

It is known that ouabain/ Na, K-ATPase signaling generates calcium oscillation through IP3R activity (Zhang et al., 2006). In study I we found that ouabain treatment can cause dephosphorylation of the ubiquitously expressed IP3R type 3 (IP3R3) at S1832 site. Structural information of the IP3R showed that dephosphorylation on S1832 may be responsible for the IP3R structural rearrangement that facilitates the opening of the calcium channel. The stromal interaction molecule 1 (STIM1) was phosphorylated on S575 upon ouabain treatment and phosphorylation on this site can activate STIM1 activity: sensing the decreased level of calcium in endoplasmic reticulum following calcium depletion via IP3R and activating plasma membrane calcium channels (Lee et al., 2012; Trebak, 2012). We used a specific STIM1 inhibitor and found that inhibition of STIM1 abolished ouabain induced calcium oscillation. Combining this new discovery with our previous findings, we can

conclude that both IP3R and STIM1 are necessary for calcium oscillatory signals generated by ouabain/ Na, K-ATPase.

CaMK2 is sensitive to the frequency of calcium oscillations and can decode the calcium frequency into its kinase activity (De Koninck & Schulman, 1998). Here we found that ouabain treatment can phosphorylate CaMK2G on S402 and S449, whose functions have not been reported before. As ouabain is anti-apoptotic and CaMK2G was also reported to be anti-apoptotic (Hojabrpour, Waissbluth, Ghaffari, Cox, & Duronio, 2012), we hypothesized that ouabain's anti-apoptotic effect may be dependent on CaMK2G activity and phosphorylation on either S402 or S449 can activate CaMK2G. Further evidence was gathered by experiments where we used siRNA to down-regulate CaMK2G gene expression in PTC. Down-regulation of CaMK2G abolished ouabain's protection from apoptosis.

In summary, this study shows that ouabain/ Na, K-ATPase generates large-scale signaling pathways that cover multiple aspects of cellular function, from cell junction to proliferation, as well as protection from apoptosis.

4.2 Study II

4.2.1 PTC, podocytes and mesangial cells express different glucose transports

SGLT2 is reported to be expressed in the S1 segments of proximal tubules, while SGLT1 is expressed in the later S3 segment of proximal tubules (Hummel et al., 2011). The expression of SGLTs in mesangial cells is controversial as some group did not detect SGLT1 gene expression in rat mesangial cells (Inoki, Haneda, Maeda, Koya, & Kikkawa, 1999), while one group showed that both SGLT1 and SGLT2 were expressed in rat mesangial cells (Wakisaka, He, Spiro, & Spiro, 1995; Wakisaka, Nagao, & Yoshinari, 2016). To confirm the SGLT expression in our primary cell culture of PTC, mesangial cells and podocytes, we performed RT-PCR analysis and found that PTC expressed both SGLT1 and SGLT2 genes, mesangial cells expressed only SGLT1 while podocytes expressed neither of the transporters. The primers we used were different from what was previously used, and the specificity of primers was carefully checked. The presence of different glucose transporters was further confirmed by experiments on glucose uptake: PTC and mesangial cells had sodium dependent glucose uptake, but this was not the case for podocytes. The percentages of sodium dependent glucose uptake were approximately 60 % and 40% in PTC and mesangial cells respectively (Figure 8). This is not surprising as SGLT2 transport two Na^+ while SGLT1 transport only one Na^+ during one transport cycle and both cells express GLUTs in their basolateral membrane (Haneda, Koya, Isono, & Kikkawa, 2003; Wright et al., 2011).

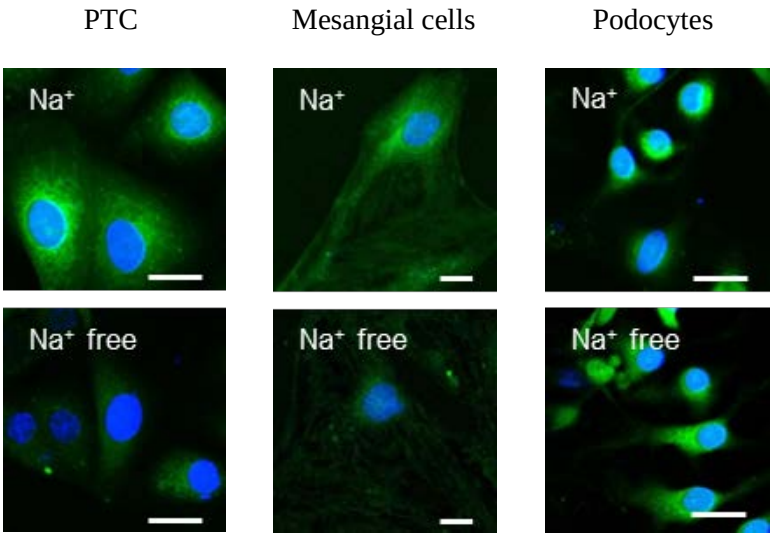

Figure 8: glucose uptake under Na^+ or Na^+-free conditions in primary PTC, mesangial cells and podocytes. Fluorescent 2-NBDG (green), a D-glucose analog, was used as an indicator of glucose uptake. The nuclei were stained with NucBlue (blue).

4.2.2 SGLT is responsible for apoptotic response to high glucose

Hyperglycemia is responsible for diabetic complications (Brownlee, 2001) and in study II we applied 10 mM or 15 mM glucose to primary PTC, mesangial cells and podocytes. These two concentrations of glucose can be seen in diabetic patients with poor glucose control (American Diabetes, 2010). PTCs were the most vulnerable renal cell to high glucose treatment as a significant increase of TUNEL stained cells was seen after 2 hours treatment. Mesangial cells responded to 15 mM glucose after 8 hours while podocytes were resistant to high glucose. No significant increase of apoptosis was seen in podocytes treated as much as 30 mM glucose for 24 hours. The number of proximal tubules with apoptosis was three times more in kidney biopsies from diabetic patients than that from healthy donors.

PTC and mesangial cells were rescued from apoptosis when they were co-treated with SGLT2 inhibitor dapagliflozin and SGLT1 and SGLT2 inhibitor phlorizin respectively. Furthermore, PTC transfected with SGLT2 siRNA became resistant to high glucose induced apoptosis.

4.2.3 Ouabain can protect from high glucose induced apoptosis via acting on Bcl-2 family

The rescuing effect of ouabain has been seen both *in vivo* (Burlaka et al., 2016; Li et al., 2010) and *in vitro* (Burlaka et al., 2013; Li et al., 2006). Here we found that ouabain can protect primary renal cells from high glucose induced apoptosis. High glucose induced apoptosis by disrupting the balance of two Bcl-2 family members Bcl-xL and Bax on mitochondria. We

found that after 8 hours high glucose treatment, the pro-apoptotic Bax protein increased its presence on mitochondria while the anti-apoptotic Bcl-xL diminished its presence. Ouabain rescued high glucose induced apoptosis by restoring the balance of Bax and Bcl-xL on mitochondria.

4.3 Study III

4.3.1 High glucose, an apoptosis inducer

To study the apoptosis process, high glucose was used as an apoptosis trigger in study III. We found that high glucose induced apoptosis on PTC in a dose and time dependent manner. In figure 9a, various glucose concentrations were used: 5 mM, 10 mM, 20 mM and 30 mM; in figure 9b, PTC were treated with 20 mM glucose for 2h, 4h and 6h. Apoptosis was detected by TUNEL assay and apoptotic index was measured as the ratio of apoptotic cells to total cell numbers.

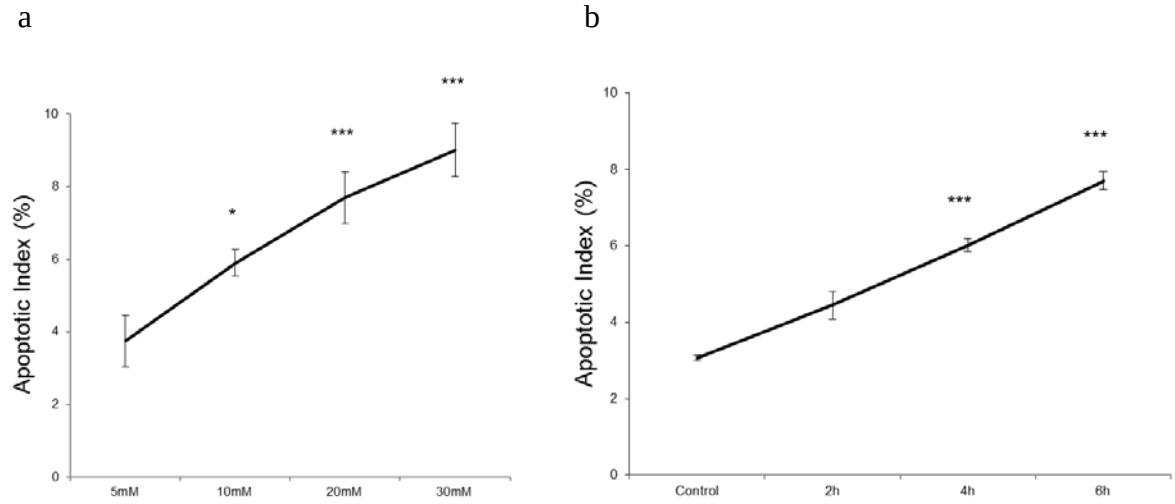

Figure 9a, b: High glucose induced apoptosis in a dose and time dependent manner. In Figure 8a, 30 mM glucose treatment for 6h increased apoptosis by 1 fold on primary PTC. In Figure 8b, 4h treatment of 20 mM glucose already significantly increased apoptosis in primary PTC compared to control.

In fact, 20 mM glucose caused a series of negative events at a rather early stage in PTC. Using JC-1 dye to measure Ψm, we observed that 1h high glucose treatment already decreased the membrane potential by 30%. Increase of intracellular ROS could also be detected after 2h treatment. Mitochondrial morphology can indicate mitochondria health status as healthy mitochondria present as an elongated and oval shape, while under toxic conditions, mitochondria turned to short and rounded shapes (Karbowski & Youle, 2003). In

this study, we found that a mitochondrial morphological change occurred after 2h high glucose treatment on PTC.

4.3.2 Mapping Bcl-2 proteins during early apoptosis with super resolution microscopy

Bcl-xL, Bax and BAD are important regulators of intrinsic apoptosis and they play distinct roles during the process. Bax are the executors of apoptosis which form pores on the MOM. Bcl-xL protect against apoptosis by inhibiting Bax. BH-3 only protein BAD bind to Bcl-xL and inhibits its action on Bax (Dewson & Kluck, 2009). In order to visualize the apoptosis initiation status, we applied super resolution microscopy – STED (stimulated emission depletion) microscopy to study Bcl-xL, Bax and BAD interaction on mitochondria in the early time points of high glucose treatment.

After 2 h high glucose exposure, BH-3 protein BAD, which remained in the cytoplasm in healthy cells, migrated to mitochondria (Figure 11). Nearest neighbor analysis (Figure 10) was performed to calculate protein-protein distances. In this case the distance between Bcl-xL and BAD became shorter after 2 h high glucose treatment (Figure 11) indicating that BAD interacted with Bcl-xL and inhibited its anti-apoptotic function.

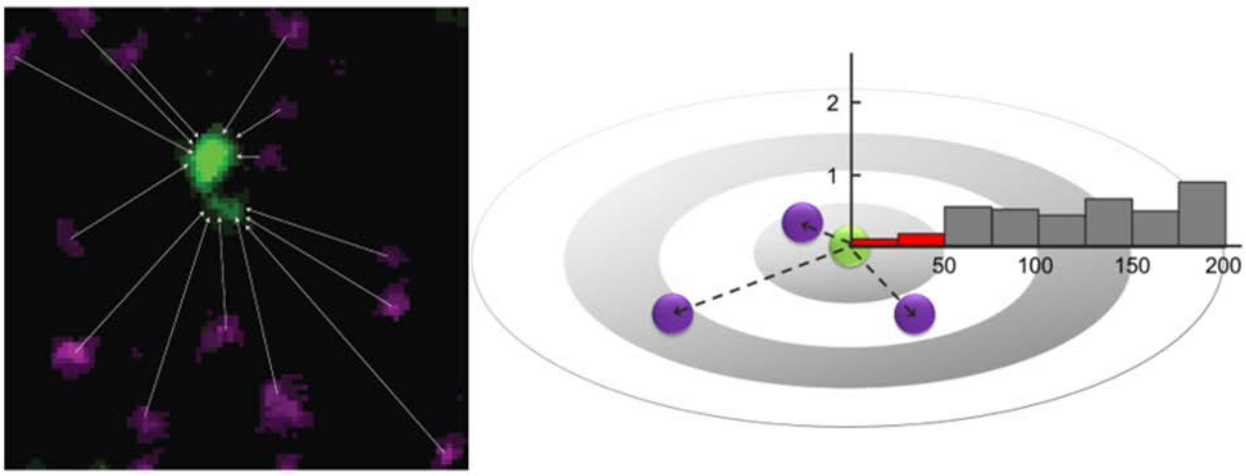

Figure 10: Schematic graphs explained the nearest neighbor analysis. The green molecule and magenta molecule represent two different proteins of interest. The distances of all the nearest magenta molecules to the green molecule were analyzed and a histogram showed fractions of different distance ranges. Distances shorter than 50 nm (shown in red panels) indicated possible interactions between the two proteins.

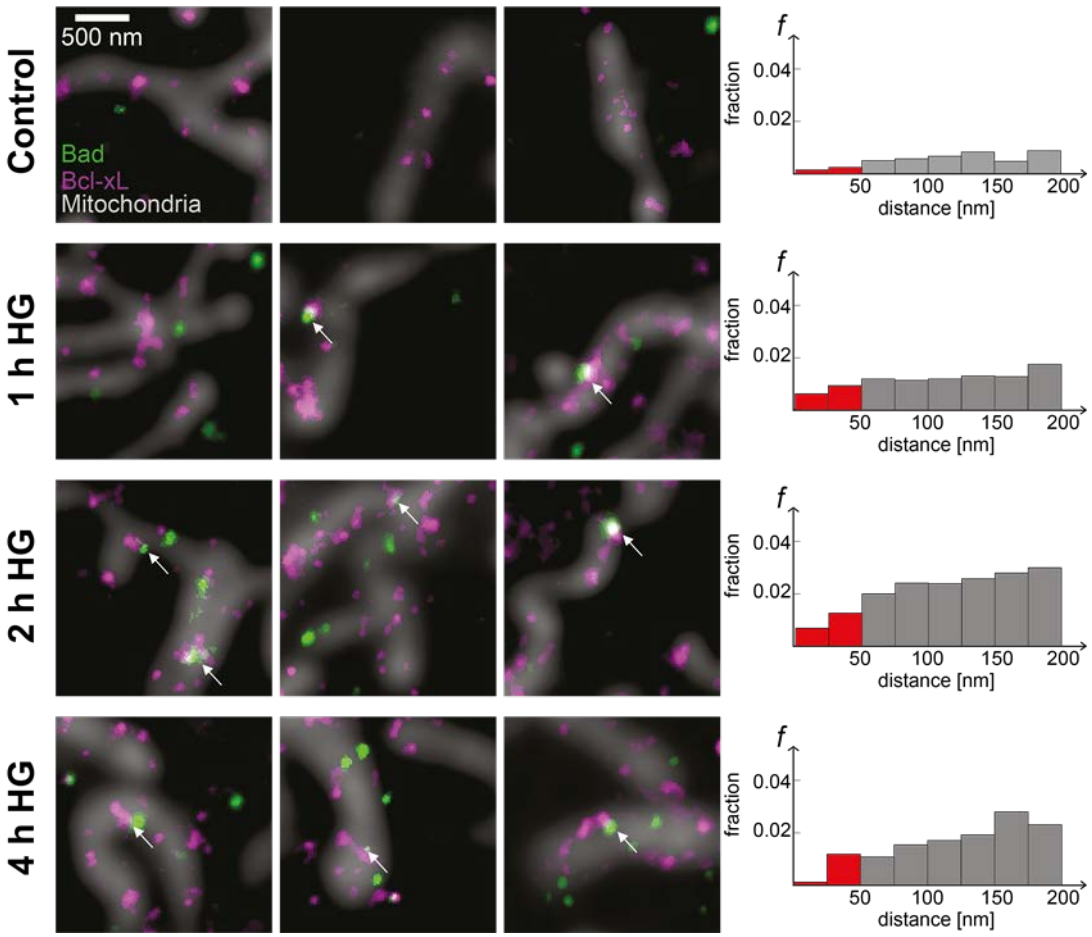

Figure 11: Histogram of 2h HG showed that after 2h high glucose treatment, significance migration of BAD to mitochondria was observed. BAD and Bcl-xL were shown in green and magenta color respectively. The mitochondria were in white color and white arrows pointed out co-localizations of BAD and Bcl-xL protein. Histograms showed the fraction of Bcl-xL distances to BAD with nearest neighbor analysis and red panels indicated possible interaction between BAD and Bcl-xL. HG: high glucose 20 mM.

After 4 h the apoptotic Bcl2 protein Bax translocated to the mitochondria where it showed more interaction with Bcl-xL by nearest neighbor analysis (Figure 12). This can be explained such that Bcl-xL were inhibited by BAD and the inhibited Bcl-xL failed to retro-translocate Bax from the mitochondria into the cytosol (Edlich et al., 2011).

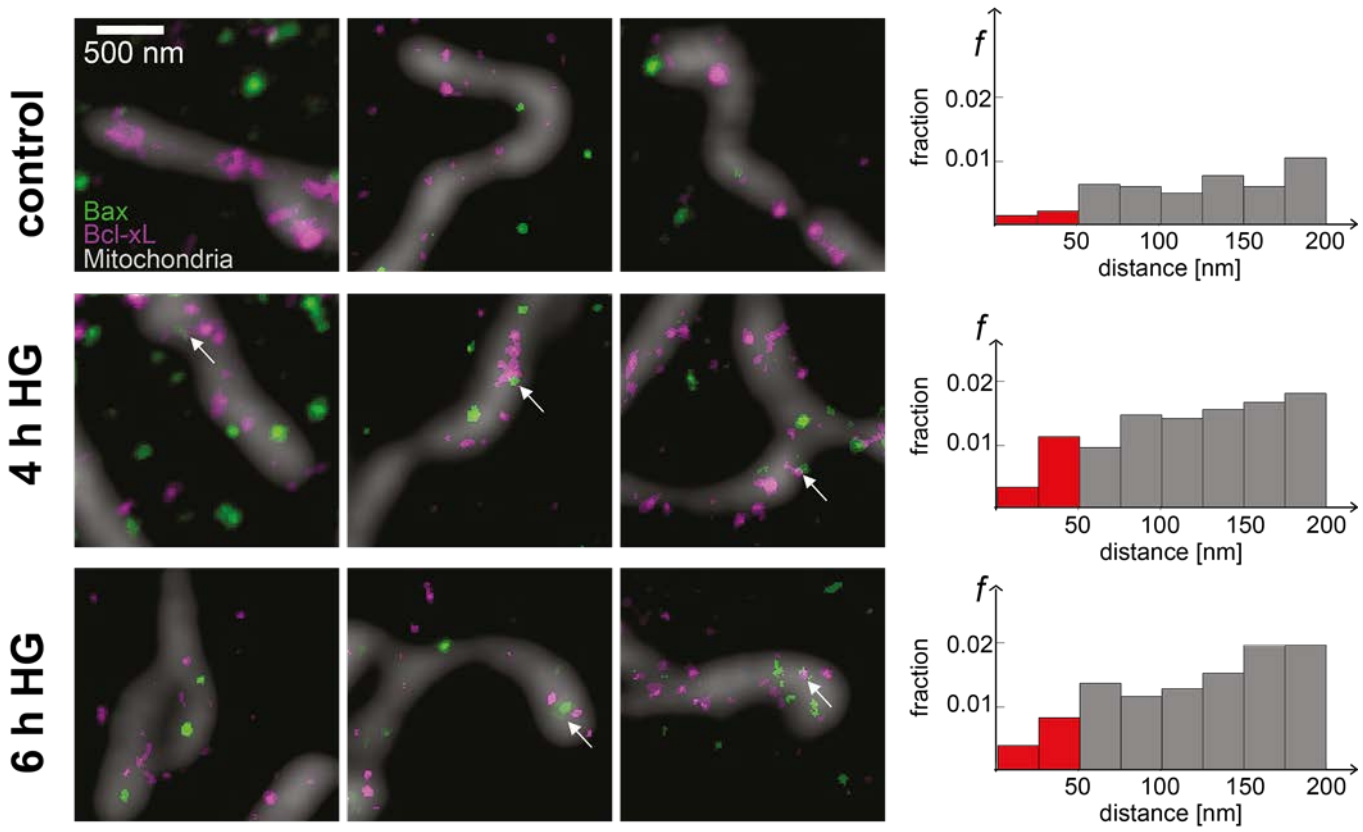

Figure 12: Histogram of 4 h HG showed that 4h high glucose exposure increased Bax and Bcl-xL interaction on mitochondria. Bax and Bcl-xL were shown in green and magenta color respectively. The mitochondria were in white color and white arrows pointed out co-localizations of Bax and Bcl-xL protein. Histograms showed the fraction of Bcl-xL distances to Bax with nearest neighbor analysis and red panels indicated possible interaction between Bcl-xL and Bax. HG: high glucose 20 mM.

The VDAC is membrane protein integrated into the MOM and has a critical role in apoptosis. Studies have shown that Bax and VDAC together can form large pores on mitochondria facilitating cytochrome c release (Martel, Wang, & Brenner, 2014; Shimizu et al., 2000; Tsujimoto & Shimizu, 2000). To study Bax and VDAC interaction, we performed two-color immunostaining of PTC and applied stochastic optical reconstruction microscopy (STORM). STORM has a higher spatial resolution than STED, but there are less available compatible dyes for STORM (Tam & Merino, 2015). After 6h high glucose exposure, Bax and VDAC had an increased interaction on mitochondria (with nearest neighbor analysis) (Figure 13) and clusters of Bax and VDAC were also observed on mitochondria. These results suggested that Bax and VDAC may form pores on the MOM after high glucose treatment which can initiate the cytochrome c release.

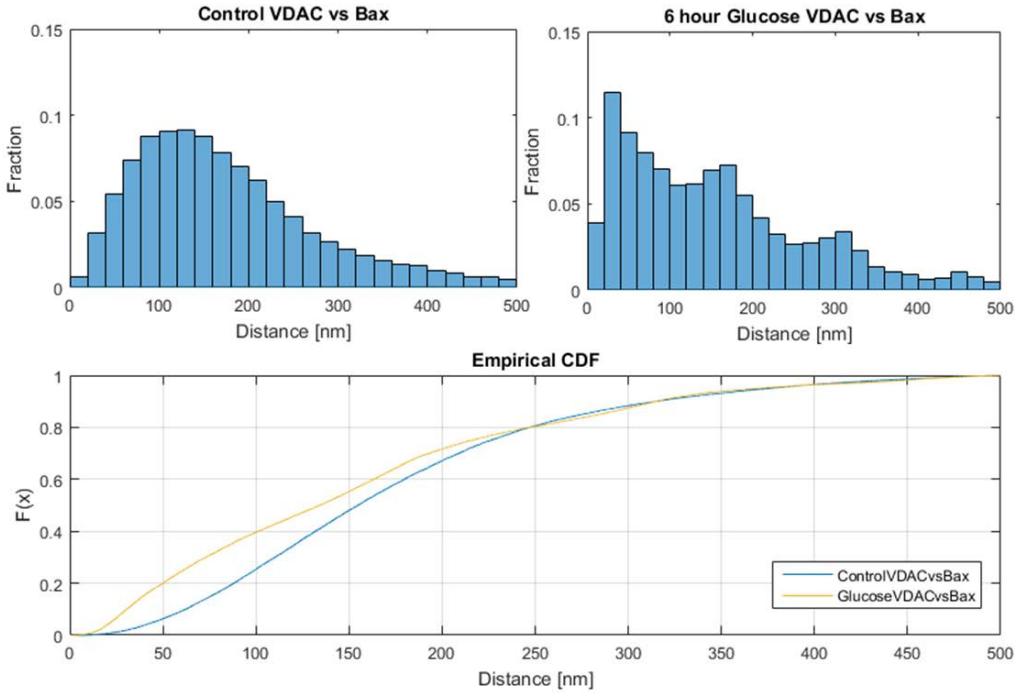

Figure 13: STORM imaging results showed increased interaction between Bax and VDAC on mitochondria after 6 hour high glucose treatment. The top-left panel showed the histogram of control sample, while the top-right panel showed the histogram of 6 hour high glucose treatment sample. Empirical CDF (Cumulative Distribution Function) analysis (the bottom panel) showed the cumulative fraction of distances between Bax and VDAC. Under control conditions, less than 10% of Bax and VDAC were at the distance shorter than 50 nm, however, 6h high glucose treatment increased the fraction to 20% which indicated increased Bax and VDAC interaction.

4.3.3 Ouabain induces CaMKK1 (CaMK2G)/Akt/BAD rescuing signaling

When 10 nM ouabain was applied together with high glucose, PTCs were rescued from high glucose induced apoptosis. Ouabain also restored Ψm and decreased ROS production. STED and STORM imaging confirmed this rescuing effect: ouabain prevented BAD migration to mitochondria and decreased interactions between BAD and Bcl-xL, Bax and Bcl-xL, Bax and VDAC. These results indicated that ouabain rescuing signaling is dependent on BAD inactivation. To further study the mechanism behind BAD inactivation, we started by checking if protein kinase B (Akt) was involved. It has previously been reported that Akt can phosphorylate BAD at S136 which stabilizes BAD docking with 14-3-3 in the cytosol (Datta et al., 1997). When we applied the Akt inhibitor to high glucose medium together with ouabain, the ouabain failed to protect PTC from apoptosis. Increased BAD migration to the mitochondria and Bax accumulation on mitochondria was also observed in the presence of the

Akt inhibitor. We further performed a Co-IP experiment and found that ouabain can restore the interaction between BAD and Akt in cytosol.

Taken together our results show that BAD and Akt are the major players in ouabain's rescue signaling. In study I, we showed that ouabain can activate CaMKK1 (Ca^{2+}/calmodulin-dependent protein kinase kinase 1) and both CaMKK and CaMK2G have been reported to activate Akt and inhibit BAD's function (Hojabrpour et al., 2012; Yano, Tokumitsu, & Soderling, 1998). We chose to further explore whether CaMKK1 is involved in ouabain rescuing signaling. When CaMKK1 was down-regulated by siRNA in PTC, ouabain lost its capacity to protect from apoptosis. In study I, we showed that when CaMK2G was silenced, ouabain also failed to protect cells from apoptosis. Taking these results together, we can conclude a new rescue signaling pathway where the calcium sensitive CaMKK1 and CaMK2G are activated by the calcium signal induced by ouabain/ Na, K-ATPase/IP3R interaction. CaMKK1 and CaMKK2G can then both activate Akt, which phosphorylates and deactivates BAD, thus protect cells from apoptosis.

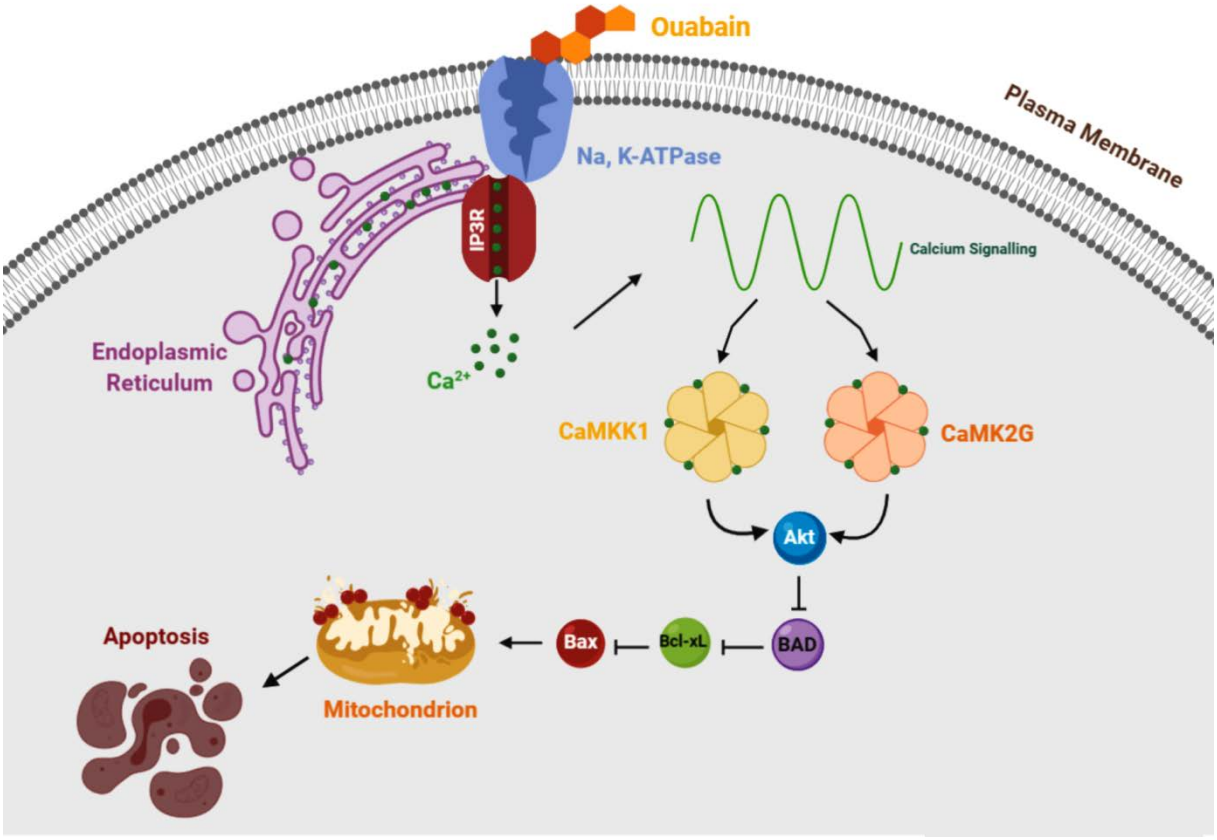

Figure 14: Ouabain induced rescuing signaling in PTC. The calcium signaling triggered by ouabain/Na, K-ATPase/IP3R interaction activates CaMKK1 and CaMK2G, both of which activate Akt and inhibit the proapoptotic effect of BAD. This signaling pathway protects cells from apoptosis. The figure is designed using Biorender online tool: https://biorender.com/.

5 General discussion and future perspectives

5.1 Cell-type targeted treatment in diabetic nephropathy

Hyperglycemia has been shown to be detrimental to renal cells both *in vivo* and *in vitro*. In diabetic patients, increased apoptosis is observed in proximal tubular cells, mesangial cells and podocytes (Verzola et al., 2007). *In vitro* studies showed high glucose can induce apoptosis in PTC (Ortiz et al., 1997), mesangial cells (Kang et al., 2003) and podocytes (Bose, Almas, & Prabhakar, 2017). In study II, SGLT expressing primary PTC and mesangial cells underwent apoptosis after high glucose treatment while primary podocytes were resistant to high glucose. This is in contradictory to other studies where podocytes underwent apoptosis from high glucose (Bose et al., 2017; Eid et al., 2009). However, the podocyte cultures used in these studies were cell lines, which compared to primary cell cultures, are functionally less representative of the cells in vivo (Pan, Kumar, Bohl, Klingmueller, & Mann, 2009). This may explain why podocyte cell lines respond to glucose in a different way from primary podocytes. Our study indicated that hyperglycemia may not be the direct cause of podocyte loss, at least not at an early stage, in diabetes.

SGLT inhibitors rescued PTC and mesangial cells from high glucose induced apoptosis in our study. In fact SGLT2 inhibitors have been used clinically as hypoglycemic agents. It has been shown that SGLT2 inhibitors are renal protective in addition to having a hypoglycemic effect: SGLT2 can slow the progression of DN and reduce clinical renal events (Neal et al., 2017; Wanner et al., 2016). More recent clinical trials showed that canagliflozin (SGLT2 inhibitor) lowers the risk of kidney failure and cardiovascular events in patients with type 2 diabetes (Perkovic et al., 2019). Among diabetic patients who had a risk for atherosclerotic cardiovascular disease, treatment with dapagliflozin (SGLT2 inhibitor) resulted in a lower rate of cardiovascular death or hospitalization for heart failure (Wiviott et al., 2019). Some of the side effects of SGLT2 inhibitors are urinary tract infection due to glucosuria and decreased bone density probably caused by increased phosphate absorption in the renal tubules (Marin-Penalver et al., 2016; Taylor, Blau, & Rother, 2015).

Study II suggested that PTC and mesangial cell death in DN is directly caused by hyperglycemia while podocytes die from other causes. It has been reported that podocytes can sense insulin levels and insulin resistance has been suggested to be responsible for podocyte dysfunction (Fornoni, 2010; Welsh et al., 2010). Therefore, DN treatment with a focus on insulin resistance may protect podocytes, while strict glucose control, such as the use of SGLT2 inhibitors, may be beneficial to PTC and mesangial cells.

5.2 Ouabain regulated phosphoproteome, a treasure to explore

In study I we identified 2580 ouabain-regulated phosphorylation events corresponding to 1242 proteins. While proteomics results showed that only 80 and 48 proteins were regulated after 10 mins and 20 mins respectively. This can be explained such that 20 mins is a rather short time compared to the half-lives of the majority of cellular proteins (Mathieson et al., 2018). The phosphorylation regulation is rather versatile as different phosphorylation patterns were seen after 10 and 20 mins. The phosphorylation events may also be initiated earlier than our experimental time point, 10 minutes. For example, ERK1/2 was detected as dephosphorylated in phosphoproteomics after both 10 mins and 20 mins. However, Western blot showed that ouabain treatment phosphorylated ERK1/2 after 5mins, and dephosphorylated ERK1/2 at 10 mins and 20 mins. Therefore, the two time points in our study are time-snaps of the dynamic phosphorylation events and important events may be missed.

We have identified phosphorylated/dephosphorylated CaMKK1 and CaMK2G and studied their anti-apoptotic function in our study, but this is only the tip of the iceberg. Study I showed that ouabain regulated phosphorylation events of proteins that regulate cell tight junction and gap junction. This confirms previous findings that ouabain can regulate gap junction and tight junction (Larre et al., 2010; Ponce et al., 2014). However, the molecular mechanism of how ouabain regulates specific proteins to affect cell junctions need to be further studied. Two other proteins that were found significantly phosphorylated were WNK1 and DNA-dependent protein kinase catalytic subunit (DNA-PKcs).WNK1 is essential for the regulation of electrolyte homeostasis and blood pressure (Wilson et al., 2001) while DNA-PKcs are involved in cell DNA repair (Chan et al., 2002). Thus ouabain's role in blood pressure regulation and cell health need to be further explored. Our study provided a database for the ouabain regulated phosphoproteome, although the biological roles of many of these regulations are yet to be fully understood and will be interesting targets for future studies.

5.3 Ouabain, a potential anti-apoptotic drug

Cell number is strictly controlled by the balance of cell proliferation and cell death. Apoptosis is the most studied programmed cell death (PCD) which plays important roles in embryo development and normal physiological function of our body. Alterations of normal apoptosis have been seen in cancer, neurological disorders, cardiovascular disorders, autoimmune diseases, and last but not lease, DN (Favaloro, Allocati, Graziano, Di Ilio, & De Laurenzi, 2012; Kumar et al., 2004). In this study we found that calcium signaling generated by ouabain/Na, K-ATPase induced CaMKK1 (CaMK2G)/Akt/BAD anti-apoptotic signaling. Studies using various animal models have showed that ouabain is protective against apoptosis in a variety of pathological conditions: traumatic brain injury, passive Heymann nephritis and adverse developmental programming of the kidney (Burlaka et al., 2016; Dvela-Levitt, Ami, Rosen, Shohami, & Lichtstein, 2014; Li et al., 2010).

In cancer cells, there is an imbalance between the pro- and anti-apoptotic Bcl-2 proteins and a defect of apoptosis. Thus some anti-apoptosis treatments may be carcinogenic (Hassan, Watari, AbuAlmaaty, Ohba, & Sakuragi, 2014). Interestingly, ouabain has an opposite effect in cancer cells compared to healthy cells. Ouabain can decrease tumor cell migration and tumor cell growth (Kometiani et al., 2005; Pongrakhananon et al., 2013). This can be explained such that ouabain can increase apoptosis in cancer cells by increasing ROS production and also through the down-regulations of the anti-apoptotic protein Bcl-2 which was shown in lung cancer cells (Pongrakhananon et al., 2013; Trenti et al., 2014).

In summary, the anti-apoptotic effect of ouabain on non-tumor cells and pro-apoptotic effect on tumor cells render it as an ideal candidate for treatment. Ouabain has been used as a cardiotonic drug to treat heart failure. However, other clinical applications of ouabain should be explored in the future as imbalanced apoptosis is a common phenomenon in diseases.

6 Concluding remarks

This thesis work has used a large scale proteomics approach to reveal the multipotent role of the ouabain/Na, K-ATPase signaling mechanism. New aspects of the signaling transducer Na, K-ATP have been discovered, opening the door for further investigation in the future. *In intro* models were applied to study the molecular mechanism behind hyperglycemia induced apoptosis, which was representative of the phenomenon observed in diabetic patients. This thesis provides a clinical guide for future DN treatment. Ouabain, a cardiotonic steroid, was shown in this thesis to rescue cells from the very early stages of apoptosis by regulating the Bcl-2 family protein, BAD. Thus there is a potential pharmaceutical use of ouabain in diseases where apoptosis is present.

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