Regulation of podocyte apoptosis in diabetic kidney disease − Role of SHIP2, PDK1 and CDK2

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ACADEMIC DISSERTATION

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To my family

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by their Roman numerals (I-III).

I Mervi E. Hyvönen, Pauliina Saurus, Anita Wasik, Eija Heikkilä, Marika Havana, Ras Trokovic, Moin A. Saleem, Harry Holthöfer, Sanna Lehtonen. Lipid phosphatase SHIP2 downregulates insulin signalling in podocytes. Molecular and Cellular Endocrinology 328(1-2):70-79, 2010.

II Pauliina Saurus, Sara Kuusela, Eero Lehtonen, Mervi E. Hyvönen, Mervi Ristola, Christopher Fogarty, Jukka Tienari, Mariann Lassenius, Carol Forsblom, Markku Lehto, Moin Saleem, Per-Henrik Groop, Harry Hothöfer, Sanna Lehtonen. Podocyte apoptosis is prevented by blocking the Toll-like receptor pathway. Cell Death and Disease, 7;6:e1752, 2015.

III Pauliina Saurus, Sara Kuusela, Vincent Dumont, Eero Lehtonen, Christopher L. Fogarty, Mariann I. Lassenius, Carol Forsblom, Markku Lehto, Moin A. Saleem, Per-Henrik Groop, Sanna Lehtonen. Cyclin-dependent kinase 2 protects podocytes from apoptosis. Submitted.

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In addition, some unpublished material is presented.

ABBREVIATIONS

AER	albumin excretion rate
AGEs	advanced glycosylation end-products
AKI	acute kidney injury
BUN	blood urea nitrogen
CD2AP	CD2-associated protein
CDK2	cyclin-dependent kinase 2
CNF	human congenital nephrotic syndrome of the Finnish type
DKD	diabetic kidney disease
DN	diabetic nephropathy
ESRD	end-stage renal disease
FSGS	focal segmental glomerulosclerosis
GBM	glomerular basement membrane
GFB	glomerular filtration barrier
GFR	glomerular filtration rate
GIT27	2-(3-Phenyl-4,5-dihydro-1,2-oxazol-5-yl)acetic acid
GLUT4	glucose transporter 4
IGF	insulin-like growth factor
IR	insulin reseptor
LPS	lipopolysaccharides
MAPK	mitogen-activated protein kinase
PA	puromycin amiononucleoside
PDK1	3-phosphoinositide dependent kinase 1
PI3K	phosphoinositide 3-kinase
PKC	protein kinase C
ROS	reactive oxygen species
SD	slit diaphragm
SHIP2	SH2-domain-containing inositol polyphosphate 5-phosphate 2
TGF-ß	transforming growth factor beta
TNF	tumor necrosis factor
TLR	toll-like receptor
TRPC6	transient receptor potential cation channel 6
T1D	type 1 diabetes
T2D	type 2 diabetes

ABSTRACT

Background: Diabetic nephropathy (DN) is a renal complication of diabetes and a common cause of end-stage renal disease (ESRD). DN presents in its earliest stage with low levels of albumin in the urine (microalbuminuria) and develops into overt albuminuria as the disease progresses. Podocyte loss due to detachment or apoptosis has a central role in the pathogenesis of DN, but the mechanisms are not fully understood. The studies in this thesis aimed to increase the knowledge of the pathophysiological mechanisms and molecular pathways leading to podocyte apoptosis in DN by studying three molecules that are expressed in podocytes, SH2-domain-containing inositol polyphosphate 5-phosphate 2 (SHIP2), 3-phosphoinositide-dependent kinase 1 (PDK1) and cyclin-dependent kinase 2 (CDK2).

Results: Lipid phosphatase SHIP2 was identified as an interaction partner of CD2AP, a protein known to be involved in the regulation of apoptosis in podocytes. We found that overexpression of SHIP2 in cultured podocytes reduced the phosphorylation of the major cell survival kinase Akt in response to insulin and increased apoptosis. We also observed that the expression of SHIP2 was upregulated in glomeruli of insulin resistant obese Zucker rats prior to the onset of proteinuria, suggesting a role for SHIP2 in the development of podocyte injury.

In contrast, we found that the expression of PDK1 and CDK2 was downregulated in obese Zucker rats before they had developed proteinuria. We also observed that treatment of human podocytes with sera from normoalbuminuric type 1 diabetic (T1D) patients with high lipopolysaccharide (LPS) activity downregulated the expression of PDK1 and CDK2. LPS treatment of mice *in vivo* also reduced PDK1 and CDK2 expression. LPS-induced downregulation of PDK1 and CDK2 was prevented both *in vitro* and *in vivo* by inhibition of the toll-like receptor (TLR) pathway using immunomodulatory agent GIT27. Knockdown of either PDK1, or CDK2 inhibited antiapoptotic Akt pathway, stimulated the proapoptotic p38 MAPK pathway and increased podocyte apoptosis.

Conclusions: Given the central role of the PI3K-dependent Akt signaling pathway in regulating cell survival, our findings suggest that SHIP2, PDK1 and CDK2 regulate apoptosis in podocytes by modulating the activity of the PI3K-dependent Akt signaling pathway.

1. INTRODUCTION

Diabetes is a chronic disease that occurs when the pancreas is no longer able to produce insulin (type 1 diabetes, T1D), or when the body cannot use the insulin it produces (type 2 diabetes, T2D). It is a global problem that is continuously increasing. T1D is usually caused by an autoimmune destruction of the insulin-producing beta cells in the pancreas. T2D, that accounts for over 90% of all cases of diabetes, is a metabolic disorder that is characterized by hyperglycemia in the context of insulin resistance and relative insulin deficiency (Kumar et al., 2015;Zimmet et al., 2001). According to International Diabetes Federation (2014), approximately 387 million people were suffering from diabetes in 2014 and the amount is estimated to increase up to 592 million by 2035. Both T1D and T2D increase morbidity and mortality due to micro- and macrovascular complications. The microvascular complications include diabetic nephropathy (DN), retinopathy and neuropathy, and the macrovascular complications refer to cardiovascular disease, stroke and peripheral vascular disease.

DN, a complication of T1D and T2D, is the most common cause of end-stage renal disease (ESRD) in patients with diabetes worldwide (Borch-Johnsen and Kreiner, 1987;Zimmet et al., 2001). DN is associated with increased risk of cardiovascular disease and mortality (Groop et al., 2009;Nathan et al., 2005). DN is characterized by increased urinary albumin excretion, glomerular hypertrophy, mesangial expansion, glomerular basement membrane (GBM) thickening, foot process effacement and podocyte apoptosis (Meyer et al., 1999;Mogensen, 1984;Mogensen and Christensen, 1984;Pagtalunan et al., 1997;Parving et al., 1982;Tervaert et al., 2010;Viberti et al., 1982).

Podocytes are highly specialized epithelial cells covering the glomerular basement membrane (GBM) with their interdigitating foot processes. Podocytes form the filtration barrier of the glomerular capillary wall together with the fenestrated endothelial cells and the GBM. Podocyte loss due to detachment or apoptosis, is one of the key features of progressive glomerulosclerosis. Podocyte loss has been reported in patients with T1D (Steffes et al., 2001) and T2D with or without DN (Meyer et al., 1999;Nakamura et al., 2000;Verzola et al., 2007), and Verzola *et al*. reported glomerular apoptosis in the kidneys of patients with DN (Verzola et al., 2007). In Pima Indians with T2D, decreasing number of podocytes per glomerulus has been shown to be the strongest indicator of the progression of the renal disease (Meyer et al., 1999).

Several key podocyte proteins essential for glomerular filtration, such as transient receptor potential cation channel 6 (TRPC6), nephrin and CD2-associated protein (CD2AP), are known to be involved

in the regulation of apoptosis in podocytes (Asanuma et al., 2007;Huber et al., 2003;Li et al., 2013). Reactive oxygen species (ROS), transforming growth factor beta (TGF-ß), angiotensin II, and lipopolysaccharides (LPS)/endotoxins (Binder et al., 1999;Chen et al., 2009;Jia et al., 2008;Li et al., 2013;Srivastava et al., 2013;Susztak et al., 2006), that are lipid-soluble membrane components of the gram-negative bacteria, have been shown to induce podocyte injury and apoptosis.

Podocyte apoptosis plays a critical role in the pathogenesis of glomerular diseases. Therefore, analysis of the molecules involved in the regulation of apoptosis in podocytes is essential to better understand the pathophysiological mechanisms of diabetic kidney diseases and to be able to prevent podocyte apoptosis and the progression of diabetic kidney complication. The studies in this thesis were initiated by characterization of new interaction partners of CD2AP, since CD2AP is known to be involved in the regulation of apoptosis in podocytes.

We found that SH2-domain-containing inositol polyphosphate 5-phosphate 2 (SHIP2), a molecule involved in insulin resistance and metabolic syndrome, interacted with CD2AP. Furthermore, SHIP2 was found to be involved in the PI3K-dependent Akt signaling pathway and regulation of podocyte apoptosis. We also identified that 3-phosphoinositide dependent protein kinase-1 (PDK1), a serine/threonine kinase that serves as a major regulatory point in Akt signaling, and CDK2, a S-phase cyclin-dependent kinase, that is regulated by Akt and also activates Akt, are involved in podocyte apoptosis. These results suggest that SHIP2, PDK1 and CDK2 regulate podocyte apoptosis by modulating the activity of the PI3K-dependent Akt signaling pathway. Therefore differences in the expression of these proteins may associate with the development of diabetic kidney diseases. Modulating the expression or functions of these molecules may provide a concept to prevent podocyte apoptosis, and could therefore be used as a treatment strategy in preventing the progression of DN.

2. REVIEW OF THE LITERATURE

2.1. Kidney structure and function

The kidneys are a pair of organs that perform several important functions in the body. They lie behind the posterior abdominal wall. The primary function of the kidneys is the filtration of plasma to secrete waste products from the blood into the urine. Kidneys play an essential role in maintaining the electrolyte, fluid and acid-base balance in the body. They also secrete hormones needed for blood pressure regulation and red blood cell formation.

Each human kidney has approximately one million nephrons, which are the basic functional units of the kidney. Each nephron consists of the glomerulus and the tubulus. The glomerulus, that is responsible of the plasma filtration, is a capillary tuft surrounded by Bowman´s capsule located in the kidney cortex (Figure 1A). The tubular system is formed by the proximal and distal tubule, and the loop of Henle (Figure 1B).

Close to 180 liters of primary urine is formed daily by the kidney glomeruli. Primary urine is modified in the tubular system by reabsorption of water and electrolytes so that the daily urinary excretion is normally 1-1.5 liters (reviewed in Tisher and Madsen, 1991).

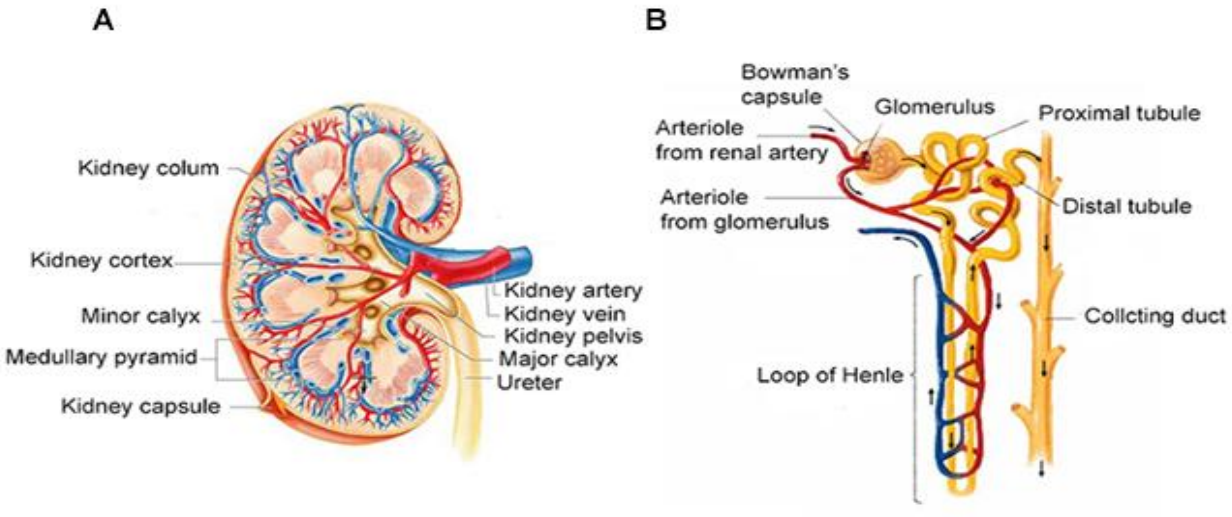

Figure 1. Illustration of kidney (**A**) and nephron (**B**) anatomy. Modified from Marieb, 2001.

2.2. Glomerular filtration barrier

The ultrafiltration of plasma occurs in glomeruli (Figure 2A), where primary urine is filtrated through the glomerular filtration barrier (GFB) (Figure 2B). Water and small and midsized solutes pass the GFB, but macromolecules and blood cells are retained in the circulation. Ultrafiltration takes place between the capillary lumen and the urinary space. GFB is a highly specialized structure forming a size, charge and shape-selective filter. The barrier consists of three layers, the fenestrated endothelial cells, glomerular basement membrane (GBM) and highly differentiated glomerular epithelial cells, podocytes that form the final filter with their slit diaphragms (Haraldsson et al., 2008).

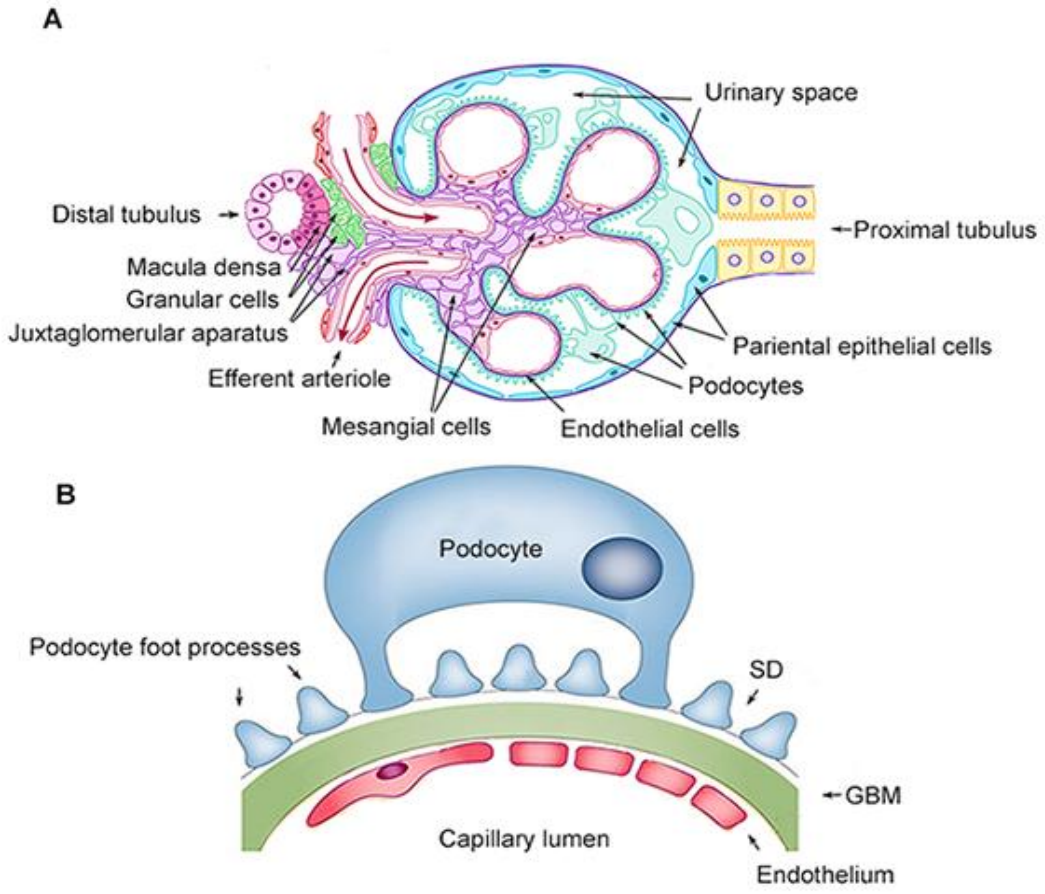

Figure 2. Structure of the glomerulus (**A**) and the glomerular filtration barrier (**B**). GMB: glomerular basement membrane; SD: slit diaphragm. Modified from Komorniczak, 2009 (**A**) and Suh and Miner, 2013 (**B**).

2.2.1. Endothelium

The glomerular endothelial cells line the glomerular capillaries forming the first part of GFB. Glomerular endothelial cell fenestrations are transcellular holes of 60-100 nm in diameter that are specialized for filtration of fluid across the cell layer (Haraldsson et al., 2008;Satchell and Braet, 2009). Glomerular filtration rate is dependent on the number and size of the fenestrations. The endothelial cells have charge-selective properties due to the negatively charged glycocalyx, that provide the first barrier to albumin (Haraldsson et al., 2008;Satchell and Braet, 2009).

2.2.2. Glomerular basement membrane

GBM is a 250-400 nm thick non-cellular layer of the glomerular filtration barrier situated between endothelial cells and podocytes. GBM is composed of extracellular matrix proteins including type IV collagen (collagen α3, α4 and α5 chains), laminins, nidogen/entactin and heparin sulfate proteoglycans, which provide an anionic charge to the GBM and form a meshwork with both size- and charge-selective properties (Haraldsson et al., 2008;Suh and Miner, 2013).

2.2.3. Podocytes

Podocytes are polarized, terminally differentiated and highly specialized cells that surround the glomerular capillaries facing the Bowman´s capsule and the primary urine and form the major component of the glomerular filtration barrier.

2.2.3.1. Slit diaphragm

The podocyte cell body gives rise to major processes that extend to foot processes which are interconnected with special cell adhesion structures called slit diaphragms (SD). Rodewald and Karnowsky (1974) were first to visualize these zipper-like structures with pores almost the same size as albumin using electron microscopy suggesting that they function as a sieve for plasma proteins (Rodewald and Karnovsky, 1974).

2.2.3.2. Podocyte proteins

There are several podocyte proteins, such as nephrin, trancient Receptor Potential Cation Channel 6 (TRPC6), CD2-associated protein (CD2AP) and podocin, known to be essential for glomerular filtration (Figure 3), that are also involved in the regulation of apoptosis in podocytes. The involvement of nephrin and CD2AP in podocyte apoptosis is described in more detail in Section 2.5.

Nephrin is the major molecule of the SD (Ruotsalainen et al., 1999). Nephrin is a transmembrane protein that belongs to the immunoglobin superfamily (Holthofer et al., 1999;Holzman et al., 1999;Kestila et al., 1998;Ruotsalainen et al., 1999). Nephrin forms the SD framework together with the Neph-protein family (Neph 1-3) (Barletta et al., 2003;Gerke et al., 2003;Ruotsalainen et al., 1999). Nephrin is mutated in the human congenital nephrotic syndrome of the Finnish type (CNF) that is a serious renal disease described already in 1956 (Ahvenainen et al., 1956;Kestila et al., 1998). Nephrin expression was found to be reduced also in human DN (Doublier et al., 2003). In addition, Li et al. (2015) demonstrated that nephrin is needed for the maintenance of the SD integrity, as mice with long-term nephrin knockout showed increase in podocyte apoptosis (Li et al., 2015).

TRPC6 that is also expressed in the SD and known to interact with nephrin (Reiser et al., 2005), was found to be mutated in focal segmental glomerulosclerosis (FSGS) (Winn et al., 2005). Moller et al., (2007) found increased TRPC6 expression in the glomeruli of patients with proteinuric kidney diseases, such as membranous glomerulonephritis and minimal-change disease (Moller et al., 2007). Lu et al. (2015) demonstrated that oxygenated podocytes undergo apoptosis due to intracellular elevation of Ca^{2+} via TRPC6 (Lu et al., 2015).

CD2AP has also been shown to interact with nephrin (Palmen et al., 2002;Shih et al., 1999;Shih et al., 2001), and it has been demonstrated that CD2AP also directly interacts with actin (Lehtonen et al., 2002). Mice lacking CD2AP develop nephrotic syndrome resembling the human disease and die of renal failure at the age of six to seven weeks. The glomeruli of these mice show podocyte effacement (Shih et al., 1999) similar to that in the CNF (Patrakka et al., 2000;Smoyer and Mundel, 1998). In addition, mutations in CD2AP have also been shown in human patients with FSGS (Gigante et al., 2009;Kim et al., 2003).

Podocin, a podocyte-associated protein, has been shown to localize to the podocyte foot process membrane and to interact with nephrin and CD2AP (Schwarz et al., 2001). Shibata et al., (2005)

demonstrated reduced expression of podocin in glomeruli of rats with PAN nephrosis (Shibata et al., 2006). In addition, mutation in podocin induces podocyte injury suggesting that podocin has a role in podocyte apoptosis (Fan et al., 2009).

In addition to nephrin, TRPC6, CD2AP and podocin, several cytosolic scaffolding molecules, tight junction proteins etc. together with the membrane proteins contribute to maintaining the structure of the SD (reviewed in Kawachi et al., 2006;Sampogna and Al-Awqati, 2012).

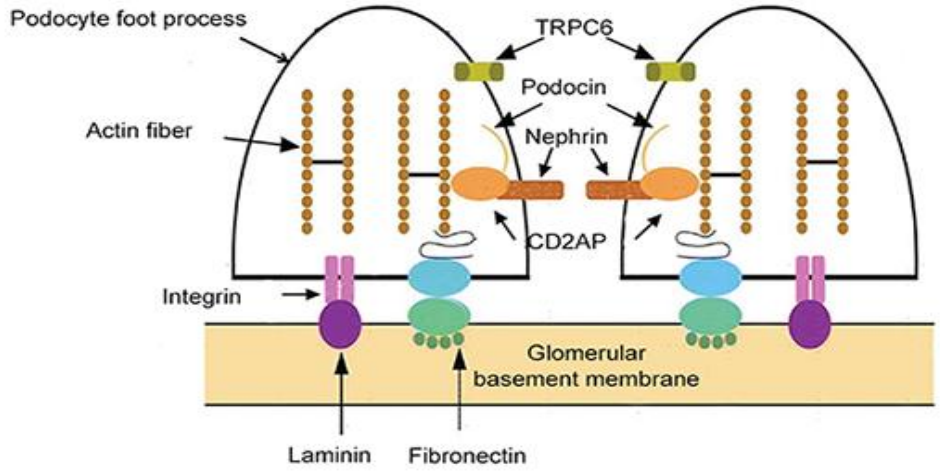

Figure 3. Schematic presentation of the molecular components of the slit diaphragm. TRPC6: Trancient Receptor Potential Cation Channel 6; CD2AP: CD2-associated protein. Modified from Dinga et al., 2015.

2.3. Diabetic Nephropathy

Diabetic nephropathy (DN) is the leading cause of kidney disease in patients starting renal replacement therapy. DN affects around 40% of type 1 and type 2 diabetic patients (Gross et al., 2005;Zimmet et al., 2001).

2.3.1. Clinical features of diabetic nephropathy

DN is a progressive kidney disease categorized into stages based on values of albumin excretion rate (AER): microalbuminuria, macroalbuminuria and proteinuria. Microalbuminuria, the early stage is characterized by a small increase in AER (30-300 mg/24 h), macroalbuminuria as AER >300 mg/24 h and proteinuria as AER >500 mg/24 h. Macroalbuminuria and proteinuria, latter classically named

overt DN, take years to develop and are considered as a definition of more advanced disease. In most cases, macroalbuminuria and proteinuria lead to decreased glomerular filtration rate (GFR) (Reutens and Atkins, 2011). DN is characterized by progression of microalbuminuria to macroalbuminuria that may subsequently lead to end-stage renal disease (ESRD). DN is the leading cause of ESRD in Europe (Kramer et al., 2009).

The increase in AER as a predictor of proteinuria in type 1 (Mogensen and Christensen, 1984;Parving et al., 1982;Viberti et al., 1982) and type 2 (Mogensen, 1984) diabetic patients, was first described in the beginning of 1980s. In these studies, it was shown that up to 85% of patients with microalbuminuria progressed to macroalbuminuria (Mogensen and Christensen, 1984;Viberti et al., 1982). However, in later studies, the risk of progression has decreased, mostly due to intensive treatment of hyperglycemia and hypertension (Caramori et al., 2000;Forsblom et al., 1992). Although considered as a risk factor for macroalbuminuria, microalbuminuria has also been shown to regress to normoalbuminuria in some patients (Perkins et al., 2003).

2.3.2. Pathology of diabetic nephropathy

DN is characterized by histological and structural changes in the kidney (Figure 4.). The glomerular classification of DN starts by GBM thickening and mesangial expansion, finally leading to advanced diabetic glomerulosclerosis (Tervaert et al., 2010). GBM thickening, the main finding of DN (Fioretto et al., 1995;Osterby, 1972) is a characteristic early change in both type 1 (Mauer et al., 1984;Osterby, 1972) and type 2 diabetic nephropathy (White and Bilous, 2000), but it does not correlate with the severity of the clinical disease (Mauer et al., 1984), since it remains stable within the duration of the disease (Fioretto et al., 1995). Mesangial expansion, defined as an increase in extracellular material in the mesangium, can be divided into mild and severe stages depending on the size of the expanded mesangial area (Tervaert et al., 2010). Mesangial expansion is a hallmark of both type 1 and type 2 diabetic nephropathy (Mauer et al., 1984;White and Bilous, 2000). Kimmelstiel and Wilson reported first in 1936 mesangial matrix accumulation as the characteristic histological feature of DN (Kimmelstiel and Wilson, 1936). They found nodular-shaped intercapillary hyalinous lesions, now often known as Kimmelstiel-Wilson lesions, as the hallmark of more severe stage of nephropathy (Hong et al., 2007;Schwartz et al., 1998;Tervaert et al., 2010). Advanced diabetic glomerulosclerosis, the most severe case of DN, is developed when Kimmelstiel-Wilson lesions finally result in glomerulosclerosis (Qian et al., 2008).

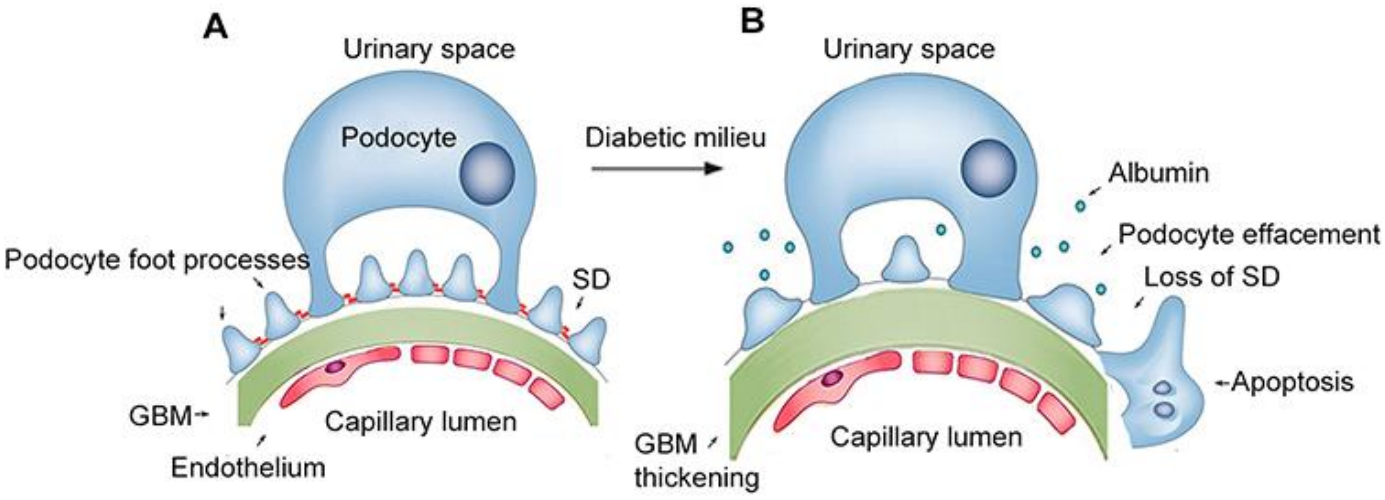

Figure 4. Characteristic changes in glomerular filtration barrier during development of diabetic nephropathy. (**A**) Normal glomerular filtration barrier. (**B**) Diabetic kidney. GBM: glomerular basement membrane; SD: slit diaphragm. Modified from Suh and Miner, 2013.

2.3.3. Mechanisms of diabetic nephropathy

Several factors affect the pathogenesis of DN. For instance age, gender, smoking, hyperglycemia, elevated blood pressure, hyperlipidemia, hyperfiltration and genetic susceptibility are risk factors identified in the development of DN (Ayodele et al., 2004;Hovind et al., 2001;Hovind et al., 2003;Zelmanovitz et al., 2009). Hyperglycemia is one of the major factors contributing to the development of diabetic kidney disease. Also, the decreased number of podocytes in glomeruli is one of the strongest predictors of progression of DN (Meyer et al., 1999;Pagtalunan et al., 1997). Renal biopsy studies in humans have demonstrated that podocytes are functionally and structurally injured in the early stages of DN (Dalla Vestra et al., 2003).

2.3.3.1. Hyperglycemia

Hyperglycemia is a crucial risk factor in the development of DN. Hyperglycemia induces the pathologic changes leading to DN, including excessive formation of advanced glycosylation end-products (AGEs), production of reactive oxygen species (ROS), increased flux through the polyol pathway and an abnormal activation of protein kinase C (PKC) as a result of increases in glycolysis

or lipid synthesis (Sheetz and King, 2002). Strict glycemic control can decrease the risk of DN (UK Prospective Diabetes Study (UKPDS) Group, 1998).

2.3.3.2. Podocyte injury

Podocytes play a critical role in maintaining the structure and function of the glomerular filtration barrier. Therefore podocyte foot process effacement and the decreased number of podocytes in glomeruli are the strongest predictors of progression of DN (Toyoda et al., 2007;Weil et al., 2012). However, understanding the pathophysiological mechanisms that underlie the loss of podocytes in DN remain limited.

2.3.3.2.1. Podocyte foot process effacement

Podocyte foot process effacement is characteristic of proteinuric renal diseases including DN (Pagtalunan et al., 1997). Widening of podocyte foot processes has been shown in patients with type 1 diabetes (T1D) and type 2 diabetes (T2D) (Meyer et al., 1999;White et al., 2002). Effacement is caused by interference with the GBM or the podocyte-GBM interaction, changes in the SD-associated proteins, abnormalities in the actin cytoskeleton and neutralization of negative cell surface charges with the apical membrane domain of podocytes (reviewed in (Mundel and Shankland, 2002).

2.3.3.2.2. Podocyte hypertrophy

Mature podocytes do not proliferate under normal conditions, however, in certain diseases such as HIV nephropathy (Barisoni et al., 1999) and collapsing glomerulopathy (Nagata et al., 1998), podocytes re-enter the cell cycle and start to proliferate. Since development of clinical nephropathy is associated with the reduction in the number of podocytes per glomerulus, and total surface area covered by podocytes does not change, this increase in the surface area must be covered by remaining podocytes (Pagtalunan et al., 1997). Due to the limited capacity to proliferate, podocytes are largely dependent on hypertrophy to increase the podocyte mass in the glomeruli (Wiggins et al., 2005). Under diabetic conditions, as mesangial cells, podocytes also undergo hypertrophic processes resulting in increased cell size (D'Agati, 1994;Petermann et al., 2002). Petermann et al. (2002) showed that mechanical stress reduces cell-cycle progression and induces hypertrophy in podocytes *in vitro*. Rüster et al. demonstrated that AGE-modified bovine serum albumin induced hypertrophy and injury in cultured podocytes which can be involved in podocyte loss (Ruster et al., 2008). In addition, Lee

et al. (2015) suggested recently that podocyte hypertrophy precedes apoptosis since inhibition of hypertrophy decreased podocyte apoptosis, whereas prevention of apoptosis had no effect on podocyte hypertrophy.

2.3.3.2.3. Podocyte loss

Podocyte loss by apoptosis, detachment or both, combined with their inability to proliferate, are key components of progressive glomerulosclerosis. Podocyte loss has been reported in patients with T1D (Steffes et al., 2001) and T2D with or without DN (Meyer et al., 1999;Nakamura et al., 2000;Verzola et al., 2007). In addition, Verzola *et al.* reported glomerular apoptosis in the kidneys of patients with DN (Verzola et al., 2007). In Pima Indians with T2D, the decreased number of podocytes per glomerulus has been shown to be the strongest predictor of the progression of the renal disease, with fewer cells predicting more rapid progression (Meyer et al., 1999). Podocytes can also be detected in the urine in patients with DN and in DN animal models, and podocyte number in urine correlates with the severity of the disease (Nakamura et al., 2000;Petermann et al., 2004).

2.3.3.3. Insulin signaling and insulin resistance in podocytes

Podocytes are insulin-responsive cells (Coward et al., 2005). Binding of insulin to its receptor on the cell surface leads to a cascade of signaling events that lead to glucose uptake into podocytes (Figure 5). Insulin signaling in podocytes is mediated via PI3K and MAPK pathways (Welsh et al., 2010). Kanai et al. (1993) demonstrated that insulin-dependent glucose uptake is mediated mainly through PI3K pathway activation, since inhibition of PI3K decreased the translocation of glucose transporter GLUT4 (Kanai et al., 1993). In addition to PI3K, the cGMP-dependent protein kinase G (PKG) pathway appears to stimulate glucose uptake in podocytes (Piwkowska et al., 2014). MAPK pathway, an alternative insulin signaling pathway, does not mediate metabolic activities of insulin, but is involved in proliferation and production of cytokines, including vasoconstrictor endothelin 1 (ET-1) (Formoso et al., 2006;Lazar et al., 1995;Tian et al., 2000). Hale et al. (2013) showed that the signaling of insulin-like growth factor II (IGF-II) in podocytes is mediated through the insulin-like growth factor receptor I via PI3K and MAPK pathways (Hale et al., 2013). In the same study, a decrease in IGF signaling resulted in podocyte death *in vitro* and in glomerular disease *in vivo* (Hale et al., 2013). In addition, mice with deletion of the insulin receptor specifically in podocytes, developed significant albuminuria pointing out the importance of insulin signaling in podocytes (Welsh et al., 2010).

The connection between insulin resistance and microalbuminuria has been shown in both diabetic (Parvanova et al., 2006;Yip et al., 1993) and non-diabetic (Palaniappan et al., 2003) individuals suggesting that insulin resistance may induce podocyte injury. Insulin resistance in podocytes was first shown in db/db mice at early stage of T2D, as podocytes from these mice were unable to phosphorylate Akt in response to insulin (Tejada et al., 2008). In addition, Mima et al. (2011) identified glomeruli as the site of insulin resistance, as streptozotocin rats with T1D and Zucker rats with T2D showed loss of insulin signaling via P13K in glomerulus (Mima et al., 2011).

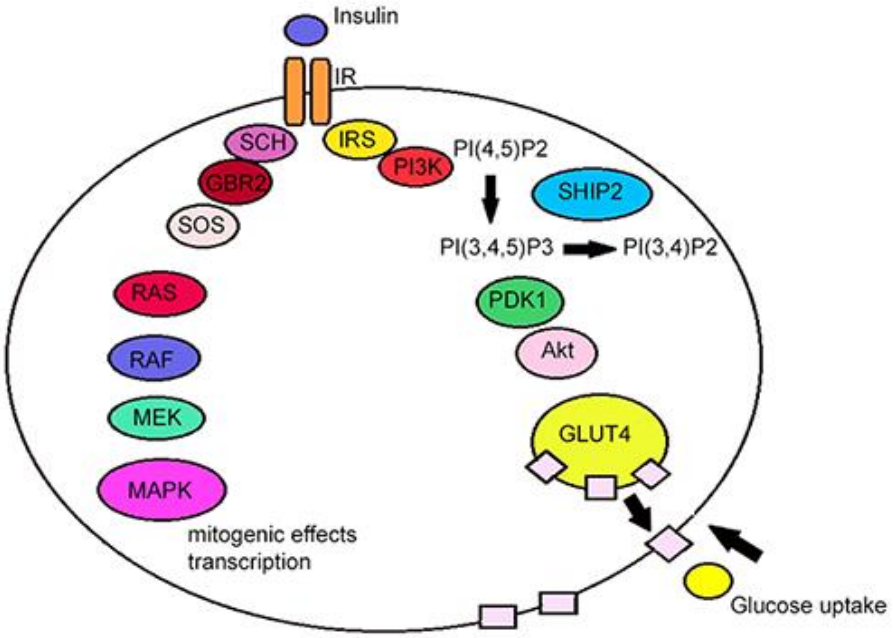

Figure 5. Schematic illustration of PI3K and MAPK insulin signaling pathways in podocytes. These pathways are differentially regulated. SHIP2 downregulates the PI3K insulin signaling pathway by hydrolyzing PIP3. IR: insulin receptor; IRS: insulin receptor substrate; PI3K: phosphoinositide 3-kinase; PDK1: 3-phosphoinositide dependent kinase; SHIP2: SH2-domain-containing inositol polyphosphate 5-phosphate 2; PI(4,5)P2: phosphoinositol(4,5)biphosphate; PI(3,4,5)P3: phosphoinositol(3,4,5)trisphosphate; (PI(3,4,)P2: phosphoinositol(3,4)biphosphate; MAPK: mitogen-activated protein kinase; GLUT4: glucose transporter 4.

2.3.4. Animal models of albuminuria and diabetic kidney disease

Animal models of albuminuria and diabetic kidney disease are needed to understand the pathogenic mechanisms of diseases such as T2D and DN, and to develop possible treatment strategies. There are multiple murine models of diabetes, however, animal disease models do not completely resemble human diseases. For example, most murine DN models develop only mild renal abnormalities when

compared to human DN. However, murine models remain popular compared to other animal models due to lower costs, large number in variety of models, the known genome and easier maintenance (Hamamdzic and Wilensky, 2013;King, 2012).

2.3.4.1. db/db mouse

The db/db mouse was identified initially in 1966 when Hummel, Dickie and Coleman found a recessive mutation in an inbred mouse strain causing diabetes (Hummel et al., 1966). Later the diabetes (db) mutation was found to encode the leptin receptor (Chen et al., 1996). The db/db mouse is a well characterized and widely used model of T2D (Sharma et al., 2003). The db/db mice are obese, insulin resistant and hyperphagic and the degree of hyperglycemia depends on the background strain (Hummel et al., 1972). The C57BLKS/J strain with diabetes is usually used (Hummel et al., 1972). Key common features of the db/db mice with the human condition are albuminuria, renal hypertrophy, GBM thickening, and mesangial expansion (Sharma et al., 2003).

2.3.4.2. Zucker rat

Obese Zucker rats (fa/fa rats) have a mutation in the leptin receptor gene (Chua et al., 1996) similar to the db/db mouse model. Leptin receptor mutations were also identified in corpulent and fatty Zucker rats, two considerably obese rodent models (Phillips et al., 1996;Wu-Peng et al., 1997). The Zucker rat is used as a model for T2D and shows similarities to early human DN (Coimbra et al., 2000), including insulin resistance, obesity, hypertension and hyperlipidemia. Renal abnormalities, including albuminuria and glomerular lesions, develop relatively late (Coimbra et al., 2000).

2.3.4.3. LPS-induced proteinuria in mice

Lipopolysaccharides (LPS) are lipid-soluble outer membrane components of the Gram-negative bacteria. LPS injection model is widely used for sepsis research. The systemic inflammation, induced by LPS administration mimics many of the initial clinical features of sepsis, including increases in proinflammatory cytokines without bacteremia (Michie et al., 1988;Remick et al., 2000;Wichterman et al., 1980). LPS also induces renal injury, including increased blood urea nitrogen (BUN), decreased GFR, and increased renal neutrophil infiltration (Cunningham et al., 2004;Knotek et al., 2001;Tiwari et al., 2005). High LPS doses induce systemic hypotension and decrease glomerular perfusion, whereas low LPS doses decrease glomerular perfusion without causing systemic hypotension (Wang

et al., 2002;Wang et al., 2003). Cani et al. (2007) reported that long-term treatment of mice with LPS causes increased inflammation and diabetes and LPS has also been shown to induce albuminuria in mice (Cani et al., 2007;Cunningham et al., 2000;Reiser et al., 2004). Therefore, LPS-induced albuminuria in mice has been used as a model to study human proteinuric kidney disease (Faul et al., 2008;Wei et al., 2008).

2.4. Podocyte apoptosis

Although the exact mechanisms causing podocyte loss in DN are not known, both apoptosis, also known as the programmed cell death, and detachment are involved (Wolf et al., 2005). Petermann et al. (2004) demonstrated that viable podocytes can be recovered from urine in rodent models, and cultured *ex vivo*, indicating that podocytes may detach without undergoing apoptosis (Petermann et al., 2004). However, there are studies that show podocyte apoptosis as a mechanism of podocyte loss in DN (Susztak et al., 2006;Verzola et al., 2007). Podocyte apoptosis increased sharply with the onset of hyperglycemia and correlated with the onset of albuminuria and preceded detectable loss of podocytes in Akita mice with T1D and db/db mice with obesity and T2D (Susztak et al., 2006). Furthermore, increased extracellular glucose concentration stimulated the generation of ROS in cultured podocytes and induced apoptosis (Susztak et al., 2006). Podocyte loss by apoptosis has also been documented as an early factor in patients with T2D (Verzola et al., 2007).

2.4.1. Anti- and proapoptotic pathways

Cell survival requires the inhibition of apoptosis, which is performed by inhibiting the expression of pro-apoptotic factors and promoting the expression of anti-apoptotic factors.

2.4.1.1. The extrinsic and intrinsic pathways

The extrinsic pathway leading to apoptosis involves transmembrane death receptors that are members of the tumor necrosis factor (TNF) receptor gene superfamily that consists of more than 20 proteins with a wide range of biological functions (Ashkenazi, 2002;Locksley et al., 2001;Walczak and Krammer, 2000). Members of this receptor family bind to extrinsic ligands and transduce intracellular signals that eventually leads to destruction of the cell (Bazzoni and Beutler, 1996; reviewed in Elmore, 2007).

The intrinsic pathway for apoptosis involves non-receptor–mediated intracellular signals, enhancing activities in the mitochondria that trigger apoptosis (Elmore, 2007). Stimuli for the intrinsic pathway include the absence of certain growth factors, hormones and cytokines, viral infections or damage to the cell by toxins, free radicals, or radiation. These stimuli cause changes in the inner mitochondrial membrane that result in the loss of mitochondrial transmembrane potential, causing the release of pro-apoptotic proteins into the cytosol and triggering apoptosis (Ashkenazi et al., 2008; reviewed in Elmore, 2007).

In the intrinsic pathway, caspases that mediate the destruction of the cell through many pathways, are activated by pro-apoptotic proteins. These pro-apoptotic proteins also translocate into the nucleus and induce DNA fragmentation, known hallmark of apoptosis (Elmore, 2007). Members of the BCL-2 family of proteins, that may be pro- or anti-apoptotic and the tumor suppressor protein p53 regulate the pro-apoptotic events in the mitochondria (Elmore, 2007).

Both, the extrinsic and the intrinsic pathways are involved in apoptosis in podocytes. For example, Eichler et al., (2006) showed toxic metals to induce podocyte apoptosis via the extrinsic pathway (Eichler et al., 2006) and Mallipattu et al. (2015) suggested regulation of Krüppel-like factor 6 (KLF6), a transcriptional factor, as a potential mechanism of the intrinsic apoptotic pathway in podocyte apoptosis (Mallipattu et al., 2015). Susztak et al. (2006) demonstrated that high glucose stimulates ROS through NADPH oxidase and mitochondrial pathways leading to the activation of proapoptotic p38 mitogen-activated protein kinase and caspase-3 and to apoptosis in cultured podocytes (Susztak et al., 2006).

2.4.1.2. Caspase cascade

Caspases are a family of cysteine proteases involved in apoptosis. Caspases are synthesized in the cell as procaspases and they are activated by cleavage with other caspases that then cleave, and thereby activate, other procaspases, resulting in an amplifying proteolytic cascade. Activated caspases then cleave target proteins at specific aspartate residues (Alnemri et al., 1996;Nicholson et al., 1995). Yuan et al., (1993) were the first to find that caspases have a role in apoptosis when a cell death-related gene, *ced-3*, which is essential for apoptosis in *Caenorhabditis elegans*, was found to be homologous to the mammalian caspases (Yuan et al., 1993). Now it is known that caspases are essential effector molecules involved in apoptosis in eukaryotic cells (Nicholson and Thornberry, 1997;Salvesen and Dixit, 1997;Thornberry and Lazebnik, 1998).

Caspases which are activated via recruitment to signaling complexes are known as the initiator caspases, since they provide a link between cell signaling and apoptosis. Caspase-2, -8, -9 and -10 are the main initiator caspases in mammals. Since most of the cellular targets are cleaved by downstream caspases, these caspases are also known as the effector caspases. The main effector caspases are caspases-3, -6 and -7 in mammals. High glucose has been shown to induce podocyte apoptosis by increased protein expression of cleaved caspase-3 in vitro (Liu et al., 2012). Similarly, Lee et al. (2015) demonstrated upregulation of cleaved caspase-3 in addition to increase in Bax/Bcl-2 ratios in vitro in high glucose-stimulated podocytes and in vivo in glomeruli of streptozotocin induced diabetic rats (Lee et al., 2015). Also, puromycin aminonucleoside (PA) induced apoptosis by increasing the activation of caspase-3 in podocytes (Li et al., 2014).

2.4.1.3. PI3K/Akt pathway

The phosphoinositide 3-kinases (PI3K) are linked to multiple cellular functions via the activation of Akt, an important player in survival signaling (Figure 6). These functions include proliferation, differentiation, cell growth, metabolism and survival (Katso et al., 2001;Saudemont and Colucci, 2009). PI3K activation has shown to play a role also in diabetic complications. Ha (2010) showed that both high glucose and advanced glycosylation end products caused podocyte hyperpermeability via PI3K/Akt signaling pathway (Ha, 2010). Bridgewater et al., (2005) demonstrated that human podocytes were protected from etoposide-induced apoptosis by insulin-like growth factors through the PI3K pathway (Bridgewater et al., 2005). Similarly, aldosterone was shown to induce apoptosis in rat podocytes through inhibition of PI3K/Akt pathway (Chen et al., 2009). Tejada et al. (2008) showed that Akt phosphorylation was downregulated in both glomeruli and podocytes of db/db mice compared to db/+ mice and podocytes (Tejada et al., 2008). Also, Akt activation has been recognized as a key survival factor for podocytes exposed to oxidized low-density lipoproteins (Bussolati et al., 2005). As well, darbapoietin was shown to protect podocytes from ultraviolet-C irradiation induced apoptosis via Akt activation (Logar et al., 2007). Therefore, loss of podocytes in early stages of DN may occur via an inability by PI3K pathway to phosphorylate Akt in response to physiological stimuli.

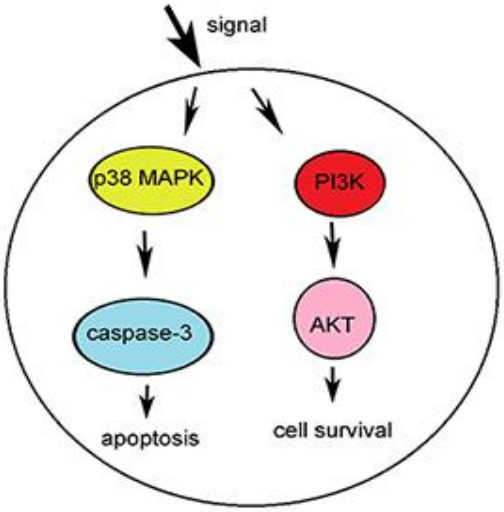

Figure 6. Schematic illustration of PI3K-dependent Akt and p38 MAPK signaling in apoptosis. Activation of p38 MAPK induces apoptosis by caspase-3 activation and PI3K activates Akt and Akt protects from apoptosis. Akt may also inhibits caspase-3 activity and therefore delay apoptosis. PI3K: phosphoinositide 3-kinase; p38 MAPK: p38 mitogen-activated protein kinase.

2.4.1.4. p38 MAPK pathway

p38 mitogen-activated protein kinase (p38 MAPK), a stress-activated serine/threonine protein kinase, belongs to the MAP kinase superfamily and it plays a major role in various cellular processes including apoptosis, cell growth, cell differentiation, inflammation, and responses to environmental stresses (Kim et al., 2012;Sayama et al., 2006). It has been shown that activation of p38 MAPK induces apoptosis in human neutrophils by activating caspase-3 and that survival signaling pathway via Akt activation inhibits caspase-3 activation and therefore delays apoptosis (Khreiss et al., 2002) (Figure 6). Verzola et al. (2007) demonstrated that p38 MAPK expression was increased in glomeruli and tubules of patients with type 2 DN, but this did not correlate with apoptosis (Verzola et al., 2007). Still, p38 MAPK activation has been suggested to be involved in apoptotic cell loss in diabetes. For instance, Adhikary et al. (2004) demonstrated that p38 MAPK signaling is increased in human and experimental DN (Adhikary et al., 2004). Also, Chen et al. (2009) demonstrated that aldosterone induced apoptosis in rat podocytes through activation of p38 MAPK pathway in addition to inhibition of PI3K/Akt pathway (Chen et al., 2009).

2.5. Anti- and proapoptotic factors in podocytes

Nephrin is known to interact with the PI3K and induce activation of the Akt signaling pathway (Huber et al., 2003). Additionally, Li et al. (2015) showed that nephrin-knockdown mice developed more podocyte apoptosis and depletion and reduced Akt phosphorylation after being subjected to doxorubicin (Li et al., 2015). In addition to nephrin, CD2AP also stimulates PI3K-dependent Akt signaling pathway that is one of the main antiapoptotic cell survival pathways (Huber et al., 2003). TGF-ß1-induced activation of Akt was shown to require CD2AP, since the lack of CD2AP enhanced the activation of the proapoptotic p38 MAPK pathway by TGF-ß1 (Schiffer et al., 2004). Nephrin and CD2AP also form a complex with dendrin that in response to podocyte injury translocates to the nucleus and enhances TGF-ß1-induced apoptosis (Asanuma et al., 2007). Peng et al. (2015) recently showed that expression of toll-like receptor 2 (TLR2) was increased in podocytes in mice with acute kidney injury (AKI) suggesting that TLR2 induces apoptosis in podocytes in AKI (Peng et al., 2015).

2.6. Induction of podocyte apoptosis

Several factors have been shown to induce podocyte apoptosis. For example, high glucose, ROS, angiotensin II, PA and LPS/endotoxins (Chen et al., 2009;Jia et al., 2008;Li et al., 2014;Li et al., 2013;Srivastava et al., 2013;Susztak et al., 2006), have been shown to induce podocyte injury.

2.6.1. High glucose

High glucose was shown increase ROS production through NADPH oxidase and mitochondrial pathways, which led to activation of p38 MAPK and caspase-3 and induction of podocyte apoptosis *in vitro* (Susztak, et al., 2006). Susztak, et al. (2006) also demonstrated increased podocyte apoptosis in Akita mice with T1D and db/db mice with T2D with onset of hyperglycemia (Susztak et al., 2006). Liu et al, (2013) showed that high glucose induces apoptosis in podocytes by upregulation of TRPC6 via ROS induction (Liu et al., 2013). In addition, high glucose has been shown to induce upregulation of TRPC6 leading to activation of the canonical Wnt signaling pathway and apoptosis in podocytes (Li et al., 2013).

2.6.2. Inflammation/Lipopolysaccharides

Sepsis, a lethal syndrome that develops in response to infection, is one of the main causes of acute kidney injury in hospitalized patients (Schrier and Wang, 2004). Lipopolysaccharides (LPS), fat-soluble outer membrane components of Gram-negative bacteria, have been shown to play a critical role in sepsis by activation of Toll-like receptor 4 (TLR4) inflammatory signaling pathways (Akira et al., 2006;Armstrong et al., 2004;Cohen, 2002;Poltorak et al., 1998). In mice, LPS-treatment induces albuminuria (Cunningham et al., 2000;Reiser et al., 2004), and long-term LPS-treatment causes increased inflammation and diabetes (Cani et al., 2007). LPS has also been shown to induce apoptosis in kidney cells and in podocytes (Cunningham et al., 2002;Guo et al., 2004;Sun et al., 2009b).

2.6.3. Puromycin aminonucleoside

PA, a classic podocyte toxin has been shown to induce podocyte apoptosis both *in vitro* and *in vivo*. Injection of PA to rats induces massive proteinuria and mimics the lesions of minimal change disease or focal segmental glomerular sclerosis (Hagiwara et al., 2006). PA induces podocyte injury, leading to foot process effacement, decreased expression of slit diaphragm proteins and actin cytoskeleton disorganization (Guan et al., 2004;Smoyer et al., 1997). PA induced podocyte injury has been shown by *in vivo* studies to be mediated via overproduction of ROS (Beaman et al., 1987;Diamond et al., 1986;Gwinner et al., 1997;Nakamura et al., 1998;Ricardo et al., 1994;Thakur et al., 1988). Xiao et al (2008) showed that vitamin D was able to reduce PA-induced podocyte apoptosis in rats (Xiao et al., 2009). Also antithrombin (Yamashita et al., 2008), triptolide (Zheng et al., 2008), upregulation of nestin (Wen, et al., 2011), dexamethasone (Wada et al., 2005) and downregulation of TRPC6 (Sun et al., 2009a) have been shown to protect podocytes against PA-induced injury and apoptosis.

3. AIMS OF THE STUDY

The mechanisms leading to the development of DN remain unclear, but podocyte injury is involved. Podocyte detachment and apoptosis are the two main factors causing podocyte loss in DN. Even though podocyte apoptosis is an early feature of DN and predicts its progression, the mechanisms and molecular pathways involved are poorly characterized. This study aimed to investigate the functions of SHIP2, PDK1 and CDK2 in the Akt cell survival pathway and in podocyte apoptosis.

The specific aims of this thesis were:

I. To study the role of SHIP2, an interaction partner of CD2AP in antiapoptotic Akt signaling pathway and in podocyte apoptosis.

II. To investigate the role of PDK1 and the molecular pathways involved in LPS-induced podocyte injury.

III. To characterize the role of CDK2 in different podocyte injury models and to investigate the signaling pathways involved.

4. MATERIALS AND METHODS

4.1. Experimental animals

4.1.1. Sprague–Dawley rats and FVB mice (Studies I-III)

The kidneys of Sprague–Dawley rats and FVB mice were used to study the expression and localization of SHIP2 and PDK1. Wild type mouse kidneys were used to study CDK2 expression and localization. The kidneys were perfused with PBS for 30 minutes at RT before collecting the kidneys.

4.1.2. db/db mice (Study I)

Male db/db mice and heterozygous db/+ controls in C57BLKS background (BKS.Cg-m+/+Leprdb/BomTac, Taconic Europe, Ry, Denmark) were used to investigate SHIP2 expression in diabetic mouse glomeruli. Blood glucose values and creatinine and urinary albumin measurements of the db/db mice are described in Study I. Animal care followed the guidelines of NIH and Finnish laws.

4.1.3. Zucker rats (Studies I-III)

Obese (fa/fa) and lean (fa/+) Zucker rats (Crl:ZUC-Leprfa) were purchased from Charles River Laboratories (Sulzfeld, Germany). Blood glucose, creatinine and urinary albumin measurements of the Zucker rats are described in Study I. Animal work was approved by the National Animal Experiment Board.

4.1.4. LPS-induced albuminuric mouse model (Studies II and III)

Female BALB-C mice (BALB/cAnNCrl) were purchased from Scanbur (KarlslundeDenmark). Mice were treated with LPS (Sigma-Aldrich, St. Louis, MO) and TLR4 inhibitor 2-(3-Phenyl-4,5-dihydro-1,2-oxazol-5-yl)acetic acid (GIT27) (Tocris Bioscience, Bristol, UK), LPS and PBS or PBS only as described in Study II. Urinary albumin and creatinine analyses were carried out at the Biochemical Analysis Core for Experimental Research (http://www.biomed.helsinki.fi/research/services/bacer/) at the Institute of Biomedicine, University of Helsinki with ADVIA 1650 Chemistry System (Siemens AG Healthcare, Erlangen, Germany) according to manufacturer's instructions. Mice were maintained

according to the principles of laboratory animal care, and the experiments were approved by the National Animal Experiment Board.

4.2. Human study subjects

4.2.1. Human kidney samples (Study II)

Kidney samples of renal cancer patients with or without T2D were obtained from surgical nephrectomies performed at Helsinki and Uusimaa Hospital district, and were from the nonmalignant part of the kidney. Albuminuria was determined from the medical records. The use of human material was approved by the local Ethics Committee.

4.2.2. Human sera (Studies II and III)

Serum samples were obtained from patients with T1D that were recruited and examined by the Finnish Diabetic Nephropathy Study (FinnDiane; www.finndiane.fi). Serum LPS activities were determined in 39 T1D patients with normal urinary albumin excretion (AER <30 mg/24 h) as described in (Nymark et al., 2009). Sera with the highest (n=6) and lowest (n=6) LPS-activities were selected for the treatment of podocytes (Study I, Supplementary Table S1). Differentiated human podocytes were treated with 10% human sera for 72 h. GIT27 (Tocris Bioscience) (10 µg/ml) was added to the media 2 h before addition of human sera with high LPS activity. The use of human material was approved by the local Ethics Committee.

4.3. Cell culture (Studies I-III)

Conditionally immortalized human podocytes were maintained as previously decribed in (Saleem et al., 2002). Shortly, cells were maintained in RPMI media supplemented with 10% fetal bovine serum (FBS), 1% glutamine, and insulin, transferrin and sodium selenite (ITS) at +33°C for proliferation. To induce differentiation, podocytes were transferred to non-permissive conditions at +37°C for 14 days (Studies I-III). HEK293FT cells (Invitrogen, Carlsbad, CA) were cultured in DMEM medium, supplemented with 10% FBS, ultraglutamine, penicillin and streptomycin. Media, FBS and ITS were obtained from Sigma-Aldrich and ultraglutamine from Lonza (Basel, Switzerland) (Studies I-III). HIRc cells (rat fibroblasts overexpressing human insulin receptor) (McClain et al., 1987) were maintained in DMEM medium, supplemented with 10% FBS, ultraglutamine, penicillin and

streptomycin (Study I). All cell culture reagents were from Sigma-Aldrich (St. Louis, MO) except ultraglutamine from Lonza.

4.4. Antibodies (Studies I-III)

The primary and secondary antibodies used in Studies I-III are presented in Table 1 and Table 2.

Table 1. Primary antibodies used in Studies I-III.

Primary antibody	**Description**	**Reference / supplier**	**Study**
SHIP2 I-20	goat polyclonal	Santa-Cruz Biotechnology (Dallas, Texas, USA)	I
CD2AP 1774	rabbit polyclonal	Lehtonen et al., 2000	I
CD2AP 1764	rabbit polyclonal	Lehtonen et al., 2000	I
phosphotyrosine PT-66	mouse monoclonal	Sigma-Aldrich	I
phosphorylated Akt (Ser473)	rabbit polyclonal	Cell Signalling Technology (Danvers, MA, USA)	I, II, III
Pan Akt	mouse monoclonal	R&D Systems (Minneapolis, MN, USA)	I, II, III
WT1	mouse monoclonal	Upstate (New York, USA)	II
caspase-3	mouse monoclonal	Cell Signalling Technology	II
podocin	rabbit polyclonal	Sigma-Aldrich	II
PDK1	rabbit polyclonal	Cell Signalling Technology	II, III
p38 MAPK	rabbit polyclonal	Cell Signalling Technology	II, III
phosphorylated p38 MAPK (Thr180/Tyr182)	rabbit polyclonal	Cell Signalling Technology	II, III
cleaved caspase-3	rabbit polyclonal	Cell Signalling Technology	II, III
active caspase-3	rabbit polyclonal	Abcam (Cambridge, UK)	II, III

BAX	rabbit polyclonal	Abcam	II, III
BCL-2	rabbit polyclonal	Abcam	II, III
CDK2	mouse monoclonal	Santa-Cruz Biotechnology	III
CDK2	rabbit polyclonal	Bethyl laboratories (Montgomery, TX, USA)	III
CDK2	rabbit polyclonal	Abcam	III
nephrin	guinea pig polyclonal	PROGEN Biotechnik (Heidelberg, Germany)	III
actin	mouse monoclonal	Sigma-Aldrich	I, II, III
tubulin	mouse monoclonal	Sigma-Aldrich	I, II, III

Table 2. Secundary antibodies used in Studies I-III.

Secondary antibody	**Description**	**Reference / supplier**	**Study**
AlexaFluor 680	goat anti-mouse	Invitrogen (Carlsbad, CA)	I, II, III
IRDye 800	donkey anti-rabbit	LI-COR (Lincoln,NE)	I, II, III
IRDye 800	donkey anti-goat	LI-COR	I, II, III
IRDye 800	donkey anti-mouse	LI-COR	I, II, III
AlexaFluor 555	donkey anti-rabbit	Invitrogen	II, III
DyLight 488	donkey anti-mouse	Jackson Immuno Research Laboratories Inc. (West Grove, USA)	II
AlexaFluor 555	goat anti-guinea pig	Invitrogen	III

4.5. Protein studies and biochemical experiments

4.5.1. Yeast two-hybrid screening (Study I)

The N-terminal part of mouse CD2AP cDNA (amino acids 1–330) was amplified by PCR and subcloned into NcoI and XhoI sites in the LexA DNA-binding domain-containing displayBAIT vector (DisplaySystem Biotech, Vista, CA). The displayBAIT-CD2AP together with the green fluorescent protein containing displayREPORTER vector were transformed into yeast strain EGY48 (Invitrogen). The rat glomerular yeast two-hybrid library (Takeda et al., 2001) in the displayTARGET vector was transformed into yeast pretransformed with displayBAIT-CD2AP and displayREPORTER vectors and plated on selective medium. Colonies expressing the green fluorescent protein reporter were selected. Plasmid DNA of the positive clones was electroporated into *Escherichia coli* KC8 (Clontech, MountainView, CA, USA) and the identity of the inserts was analyzed by sequencing.

4.5.2. Immunoblotting (Studies I-III)

Cells were washed twice with phosphate-buffered saline (PBS) and scraped from the dish into ice-cold 1% Nonidet P-40 (NP-40), 20 mM Hepes, pH 7.5, 150 mM NaCl with 1× CompleteTM, EDTA-free proteinase inhibitor cocktail (Roche), 50 mM NaF and 1 mM Na3VO4 (lysis buffer) and rotated at 4◦C for 15 min. Glomeruli and tubules were isolated from rat kidney cortices by graded sieving and lysed by rotation in lysis buffer at 4◦C for 30 min. Detergent insoluble material was removed by centrifugation (10,000×g for 10 min). For immunoblotting, proteins were separated by SDS-PAGE and transferred to PVDF-FL membranes (Millipore, Billerica, MA) and blocked with Odyssey blocking buffer (LI-COR) diluted 1:1 with PBS or TBS. Membranes were incubated for 1h at room temperature or overnight at +4°C with primary antibodies, followed by 1 h incubation with secondary antibodies. Detection and quantification was performed with an Odyssey Infrared Imager (LI-COR).

4.5.3. Pull-down assay (Study I)

Generation of glutathione-S-transferase (GST)-tag fusion protein constructs of mouse CD2AP SH3 domains is described in (Palmen et al., 2002). The N-terminal part of mouse CD2AP (aa1–330) was subcloned into pGEX-4T-1 vector (Amersham Biosciences, Buckinghamshire, England) to produce GST-CD2AP-N. GST and GST-CD2AP fusion proteins were produced in *Escherichia coli* BL21

(DE3) (Stratagene; LaJolla, CA) and purified on glutathione- Sepharose beads (Amersham Biosciences). Cell and glomerular lysates were incubated with glutathione-Sepharose beads at 4◦C for 1h, followed by incubation at 4◦C for 4h with GST-CD2AP fusion proteins or GST beads. Beads were washed with lysis buffer, boiled in Laemmli sample buffer and proteins were separated by SDS-PAGE for immunoblotting with anti-SHIP2 I-20 (Study I).

4.5.4. Immunoprecipitation (Study I)

HIRc cells in basal state, starved for 16 h, or starved for 16 h and stimulated with 17 nM insulin for 7 min were lysed. Lysates were incubated with protein A-Sepharose beads (Invitrogen) or TrueBlot anti-goat Ig IP Beads (eBioscience, SanDiego, CA) at 4◦C for 1 h, followed by incubation with anti-CD2AP 1764 (Lehtonen et al., 2000) or normal rabbit serum, or alternatively, with anti-SHIP2 I-20 or goat IgG for 16–20 h at 4 C. Protein complexes were bound to protein A-Sepharose or goat TrueBlot beads at 4◦C for 1 h, washed and boiled in Laemmli sample buffer for immunoblotting with anti-SHIP2 I-20, anti-CD2AP 1764, or anti-phosphotyrosine.

4.5.5. High glucose and insulin treatments (Studies I-III)

Human podocytes were cultured at +37◦C for 14 days in RPMI medium containing 30 mM glucose, or mannitol (osmolality control) (Studies I and II), or for 72 hours in RPMI medium containing 30 mM glucose (Study III). For insulin stimulations, differentiated podocytes or podocytes overexpressing SHIP2 were serum and ITS starved overnight and stimulated with 200 nM insulin for 15 min (Study I).

4.5.6. Transfections and infections (Studies I-III)

For SHIP2 overexpression, proliferating human podocytes were transfected for 48 h with human SHIP2 cDNA with HA-tag in pCGN vector (SHIP2-pCGN) (Dyson et al., 2001) or empty pCGN using Lipofectamine 2000 (Invitrogen). Differentiated podocytes were infected using lentiviral overexpression for SHIP2. For lentiviral overexpression, SHIP2 with HA-tag was subcloned from SHIP2-pCGN into pSIN18.cppt.hEF1αp.WPRE vector (Gropp et al., 2003). Empty pSIN18.cppt.hEF1αp.WPRE vector was used as a control. For virus production for SHIP2 overexpression, KSpCMVα8.9 and pHCMV packaging plasmids, together with SHIP2 or control lentiviral vector, were transfected into HEK293FT cells (Invitrogen) with Lipofectamine 2000. Virus-

containing media were collected 72 h later, filtrated through 0.45 µm filter, and concentrated by ultracentrifugation. Viruses were resuspended in PBS, added to podocytes on day 10 or 11 of differentiation and incubated at +37°C for 10 min, followed by centrifugation 1360×g at +4 °C for 30 min. Virus-containing medium was replaced with regular medium after 24 h (Study I).

In order to knock down PDK1 in differentiated human podocytes, lentiviral human pLKO1-shPDK1 vectors PDK1A (GAAGGTATATTAGGACATTTG) and PDK1B (TATAGACTCAGAAGGTATATT) were used. Human lentiviral pLKO1-shCDK2 vectors, CDK2A (CTCCTGGGCTGCAAATATTAT) and CDK2B (CCTCAGAATCTGCTTATTAAC) (Biomedicum Genomics, University of Helsinki, Finland) were used to knock down CDK2 in differentiated human podocytes. An empty pLKO1 vector was used as a control for both PDK1 and CDK2 knockdowns. For virus production, CMVDelta8.9 and phCMVg packaging plasmids, together with PDK1A, PDK1B, CDK2A, CDK2B or pLKO1, were transfected into HEK293FT cells (Invitrogen) with Lipofectamine2000 (Invitrogen). Media was collected and podocytes infected as described above (Studies II and III).

4.5.7. Induction of apoptosis (Studies I-III)

Apoptosis was induced by exposing differentiated podocytes to PA (50 µg/ml (Sigma-Aldrich) or LPS (100 ng/ml *Escherichia coli* 0111:B4 LPS (Sigma-Aldrich)) for 48 h. The activity of 100 ng/ml LPS in cell culture media (1.7 EU/ml) was measured as described (Nymark et al., 2009). GIT27 (Tocris Bioscience) (10 µg/ml) was added to the cells 2 h before LPS exposure.

4.5.8. Detection of apoptosis (Studies I-III)

4.5.8.1. FACS (Studies I-III)

Apoptosis was detected by fluorescence-activated cell sorting (FACS) using Annexin V-FITC Apoptosis Kit (Pharmingen, BD, Franklin Lakes, NJ) and doublestaining with 7-Aminoactinomycin D (7-AAD) (BD). Cells were trypsinized, combined with detached cells in media, and centrifuged. Cell pellet was washed with cold PBS and resuspended in Annexin V Binding Buffer (BD) containing Annexin V-FITC and 7-AAD. Cells were incubated for 15 min at room temperature in dark, washed with cold PBS and centrifuged. Infected cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA). After fixation, cells were washed with PBS, centrifuged, and

analyzed using FACSAria (BD). Cells positive for Annexin V-FITC and negative for 7-AAD were deemed apoptotic. A total 1 x 10^4 cells were detected by FACS in each sample.

4.5.8.2. In-Cell Western (Studies I-III)

Apoptosis was also detected by In-Cell Western using black 96-well plates (PerkinElmer, Waltham, MA, USA). For In-Cell Western, podocytes were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) in PBS and permeabilized with 0.1% Triton X-100 in PBS. Cells were incubated with primary antibody at room temperature for one hour, followed by secondary antibody and 1 µM DRAQ5™ (Thermo Fisher Scientific, Waltham, MA, USA) incubation at room temperature for one hour. Detection and quantification was performed with an Odyssey Infrared Imager (LI-COR). Apoptosis was also detected by immunoblotting as described in 4.5.2.

4.6. Immunofluorescence microscopy (Studies I-III)

Differentiated podocytes were fixed with 2% or 4% paraformaldehyde (Electron Microscopy Sciences) in PBS and permeabilized with 0.1% Triton X-100 in PBS. Rat kidney cryosections were fixed with acetone. Samples were blocked with CAS-block (Invitrogen), incubated with primary antibodies for one hour at room temperature (cells) or overnight at +4°C (tissue sections) in ChemMate (Dako Cytomation, Glostrup, Denmark), washed with PBS and incubated with secondary antibody and Hoechst staining DNA (Fluka, Sigma-Aldrich) for 1 h in ChemMate (Dako Cytomation). Samples were mounted in Mowiol and examined with Zeiss Axiophot 2 microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) or Leica SP2 confocal microscope (Leica Microsystems CMS GmbH, Mannheim, Germany).

For CDK2 stainings, perfused mouse kidney samples were fixed with formaldehyde and embedded in paraffin. Deparaffinized sections were blocked with CAS-block (Invitrogen), incubated with primary antibodies overnight at +4°C in ChemMate (Dako Cytomation), washed with PBS and incubated with secondary antibodies. Samples were mounted in Vectashield with DAPI (Vector Laboratories, Burlingame, CA) and examined with Leica TCS CARS SP8 confocal microscope (Leica Microsystems CMS GmbH, Mannheim, Germany) (Study III).

4.7. Immunohistochemistry (Studies I-III)

4.7.1. Immunohistochemistry of mouse kidney samples (Studies I-III)

Mouse kidney samples were fixed with formaldehyde, dehydrated and embedded in paraffin. Immunoperoxidase staining was performed with a Vecta Stain Elite ABC kit (Vector Laboratories, Burlingame, CA). Sections were deparaffinized, antigen retrieval was done by boiling for 15 min in a microwave oven in 10 mM citric acid, pH 6.0 (Studies II-III), and endogenous peroxidase was inactivated by incubation in 0.5% hydrogen peroxide in methanol for 30 min. Sections were blocked with CAS-block (Zymed, South San Francisco, CA or Invitrogen) and incubated with primary antibodies diluted in ChemMate ^{TM} (Dako, Glostrup, Denmark) and with biotinylated secondary antibodies followed by detection with VectaStain Elite ABC kit and AEC (Dako Cytomation). Slides were counterstained with hematoxylin, mounted with Shandon Immu-Mount (ThermoScientific, Waltham, MA) and photographed using Nikon Eclipse 800 microscope using the same microscope settings throughout the analysis. The staining intensity of SHIP2, PDK1 and CDK2 in mouse kidneys was visually graded by two independent researchers blinded from the treatment group.

4.7.2. Immunohistochemistry of human kidney samples (Study II)

Human kidney samples were fixed and stained as described above. Sections were counterstained with hematoxylin and photographed using Nikon Eclipse 800 microscope using the same microscope settings throughout the analysis.

4.8. Electron microscopy (Studies II and III)

Mouse kidney samples were fixed at room temperature for 2 h in 1.5% glutaraldehyde, 3% paraformaldehyde, 5% sucrose in 0.1 M cacodylate buffer, pH 7.4, postfixed in 1% osmium tetroxide (OsO4) in the same buffer for 1 h, stained en-bloc in 1% uranyl acetate in 10% ethanol for 1 h, dehydrated in ethanol and embedded in Epon. Samples were stained with uranyl acetate and lead citrate and examined with JEM-1400 Transmission Electron Microscope (Jeol, Tokyo, Japan) equipped with Olympus-SIS Morada digital camera (Olympus Soft Imaging Solutions GmbH, Münster, Germany). Foot process width was determined as described (van den Berg et al., 2004). Briefly, the number of foot processes per capillary loop was counted, divided by the length of the GBM, and multiplied by $\pi/4$. Calculations were performed on 3-4 animals per group, 3 glomeruli per

animal and the foot process width was expressed as averages of measurements of three capillary loops per glomerulus.

4.9. Statistical analyses (Studies I-III)

All variables were presented as mean ± STDEV. In all experiments, the differences between groups were determined with the Student's *t*-test (GraphPadPrism6, GraphPad Software Inc, La Jolla, CA, USA). For all statistics, p-values of less than 0.05 were considered statistically significant.

5. RESULTS

5.1. Study I: Lipid phosphatase SHIP2 downregulates insulin signaling in podocytes

5.1.1. CD2AP interacts with SHIP2 in glomeruli

CD2AP is critical to the structural and functional integrity of the glomerular filtration barrier as mice lacking CD2AP develop nephrotic syndrome with heavy proteinuria and effacement of podocyte foot processes (Shih et al., 1999). A yeast two-hybrid screening of a rat glomerular library was performed to study the role of CD2AP in podocyte injury. SHIP2, a lipid phosphatase that hydrolyzes the 5-position phosphate of phosphoinositol(3,4,5)trisphosphate (PI(3,4,5,) P_3) to generate phosphoinositol(3,4)bisphosphate (PI(3,4) P_2), and thus negatively regulates the PI3K pathway mediating various metabolic effects of insulin (Dyson et al., 2005), was found to bind to CD2AP. The function of SHIP2 in the kidney has not been studied, although SHIP2 has previously been shown to be widely expressed in different tissues (Schurmans et al., 1999). Immunoblotting of isolated rat glomeruli and tubuli was used to confirm the expression of SHIP2 in glomeruli and tubuli (Study I, Figure 1A). Immunoperoxidase staining of mouse kidney sections indicated that in the glomeruli SHIP2 localizes in podocytes (Study I, Figure 1B-D).

The yeast two-hybrid screening revealed that the C terminus of SHIP2 binds to CD2AP. Based on the sequence analysis, the COOH-terminal region contains a proline-rich domain that mediates the interaction of SHIP2 with the NH_2-terminus of CD2AP harboring the SH3 domains. A pull-down assay on mouse podocyte cell lysates with GST-CD2AP 1., 2. and 3. SH3 domains and the GST-CD2AP-NH_2-terminus was performed in order to identify, which one of the three SH3-domains of CD2AP mediates the interaction between SHIP2 and CD2AP. The pull-down assay confirmed the interaction between SHIP2 and CD2AP in glomeruli and suggested that the binding is primarily mediated by the 3. SH3-domain of CD2AP (Study I, Figure 2A and B).

A co-immunoprecipitation assay in rat fibroblasts expressing the human insulin receptor (HIRc) showed that CD2AP antibodies co-immunoprecipitated SHIP2 confirming the physiological interaction between SHIP2 and CD2AP (Study I, Figure 2C). The interaction was not dependent of insulin stimulation, since there was no difference in the interaction between basal state, starved and insulin stimulated cells. There were two separate forms of SHIP2 and only the one of lower molecular weight was precipitated by CD2AP. Immunoprecipitation with anti-SHIP2 IgG followed by Western

blotting with anti-phosphotyrosine antibodies revealed the upper band to be phosphorylated SHIP2 (Study I, Figure 2D). Interestingly, CD2AP seemed to bind only to SHIP2 in its non-tyrosine phosphorylated form.

5.1.2. SHIP2 expression and localization after insulin and high glucose treatment in cultured podocytes

To study the effect of high glucose treatment on SHIP2 expression, human podocytes were cultured in cell culture medium containing high glucose for the time of their differentiation (two weeks) or mannitol (osmotic control). SHIP2 was expressed at equal levels in both conditions, and also the Akt phosphorylation response to insulin was similar. This confirmed that SHIP2 expression level in cultured podocytes is not controlled by high glucose concentration. In basal and starved state in differentiated podocytes, SHIP2 localizes mainly in the cytoplasm, but after insulin stimulation, it was partly translocated to plasma membrane (Study I, Figure 3A-D). The cytoplasmic localization of CD2AP was not changed after insulin stimulation (Study I, Figure 3E and F).

5.1.3. Overexpression of SHIP2 downregulates insulin signaling and induces apoptosis in podocytes

Since activation of the PI3K pathway by insulin leads to phosphorylation of Akt protein kinase, the role of SHIP2 in the PI3K pathway was studied by measuring the phosphorylation of Akt in differentiated human podocytes overexpressing SHIP2. There was no difference in Akt phosphorylation in unstimulated cells when SHIP2 overexpressing cells were compared with control cells transfected with an empty vector (Study I, Figure 4A). Akt phosphorylation level remained 15.5% lower in SHIP2 overexpressing cells when podocytes were stimulated with insulin, (Study I, Figure 4B). These results were confirmed with an ELISA-based Akt activity assay. There was no difference in Akt activity after insulin stimulation in SHIP2 overexpressing cells (Study I, Figure 5A), whereas in control cells Akt activity was increased by 32% (Study I, Figure 5B). Akt activation results indicate that SHIP2 downregulates the insulin signaling pathway in podocytes.

Since PI3K-dependent Akt signaling pathway has also a protective role in regulating cell survival in podocytes, we next studied the effect of SHIP2 overexpression on apoptosis. SHIP2 overexpression increased apoptosis in differentiated human podocytes to 8.6% when compared to control podocytes

infected with empty vector showing an apoptosis rate of 4.5%, indicating that SHIP2 has a role in regulating podocyte apoptosis (Figure 7 and Study I, Figure 6).

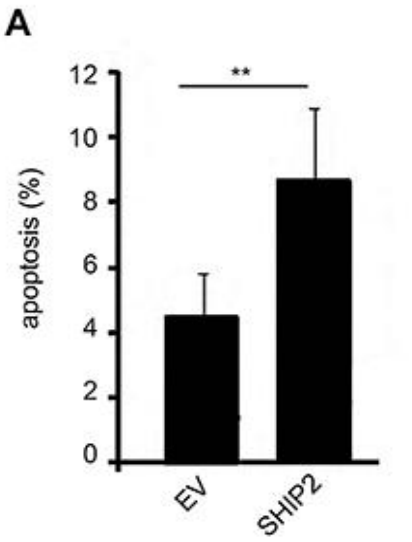

Figure 7. SHIP2 overexpression increases apoptosis in cultured podocytes. **A:** Flow cytometry of podocytes stained with Annexin V shows that SHIP2 overexpression increases podocyte apoptosis compared to podocytes infected with empty vector (EV). The experiments were performed three times with three replicates in each experiment. The bars (**A**) show the mean expression in arbitrary units (error bars STDEV). $^{**}p < 0.01$, Student's *t*-test.

5.1.4. SHIP2 is upregulated in glomeruli of diabetic rats and mice

Zucker rats and db/db mice that are both insulin resistant and slightly diabetic due to a mutation in the leptin receptor gene (Chua et al., 1996), were used to study the expression of SHIP2 *in vivo*. In Zucker rats, kidneys were analyzed from three lean and three obese rats at 12 weeks of age and from six lean and six obese rats at 40 weeks of age. 40 weeks old obese rats had developed proteinuria, as the urine albumin/creatinine ratio was higher in obese rats than in controls. These rats had also slightly higher weights and blood glucose levels than the controls, but these differences were not statistically significant. Both age groups showed upregulation of SHIP2 expression in obese (fa/fa) rats when compared to lean controls (fa/+) (Study I, Figure 7).

db/db mice also had notable albuminuria at the age of 8 weeks and were highly hyperglycemic and clearly obese compared to heterozygous db/+ controls (Study I, Table 1). SHIP2 expression level in immunoperoxidase stained kidney sections was analyzed by two independent researchers blinded from the genotypes in eight glomeruli from each sample. Although there was a trend of higher expression of SHIP2 in db/db mice when compared to db/+ controls, it was not significantly increased (Study I, Figure 8).

5.1.5. SHIP2 is upregulated in PA-induced podocyte injury *in vitro*

PA is a toxin that induces experimental minimal change nephrosis in rats (Marshall et al., 2006). PA is also known to induce podocyte injury and apoptosis both *in vitro* and *in vivo* (Srivastava et al., 2013;Sun et al., 2009a;Xiao et al., 2009). PA-treatment of differentiated human podocytes increased the expression level of SHIP2 and induced apoptosis 48 hours after exposure (Figure 8).

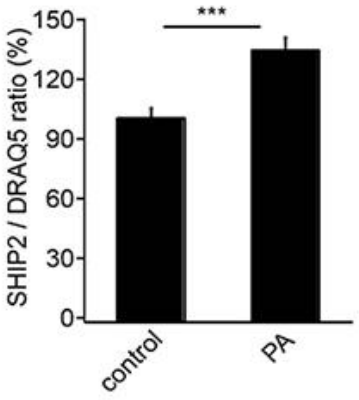

Figure 8. SHIP2 expression is upregulated in human podocytes treated with PA. **A**: Quantification of In-Cell Western of SHIP2 in podocytes treated with PA shows that the expression of SHIP2 is higher after PA- treatment. DRAQ5^{TM} was used for normalization.The experiment was performed three times with 24 replicates per group. The bars (**A**) show the mean expression in arbitrary units (error bars STDEV). ***$p < 0.001$, Student's *t*-test.

5.2. Study II: Podocyte apoptosis is prevented by blocking the Toll-like receptor pathway

5.2.1. PDK1 expression is reduced and apoptosis increased in cultured human podocytes treated with sera with high LPS activity from T1D patients

PDK1 is a serine/threonine kinase known to play an essential role in Akt signaling in various cell types, but its function in podocytes hasn't been studied before. To study the role of PDK1 in podocytes, we first confirmed that it is expressed in glomeruli and tubuli by immunoblotting of isolated rat glomerular and tubular fractions (Study II, Figure 1A). Expression of PDK1 in the nuclei of podocytes was confirmed by double labeling of rat kidney sections with PDK1 and Wilms tumor 1 (WT1) antibodies (Study II, Figure 1D-F). In addition, double labeling of normal rat kidney sections with PDK1 and nephrin antibodies confirmed that PDK1 is expressed in the cytoplasm of podocytes (Figure 7). PDK1 is also detected in other glomerular cells (Figure 9).

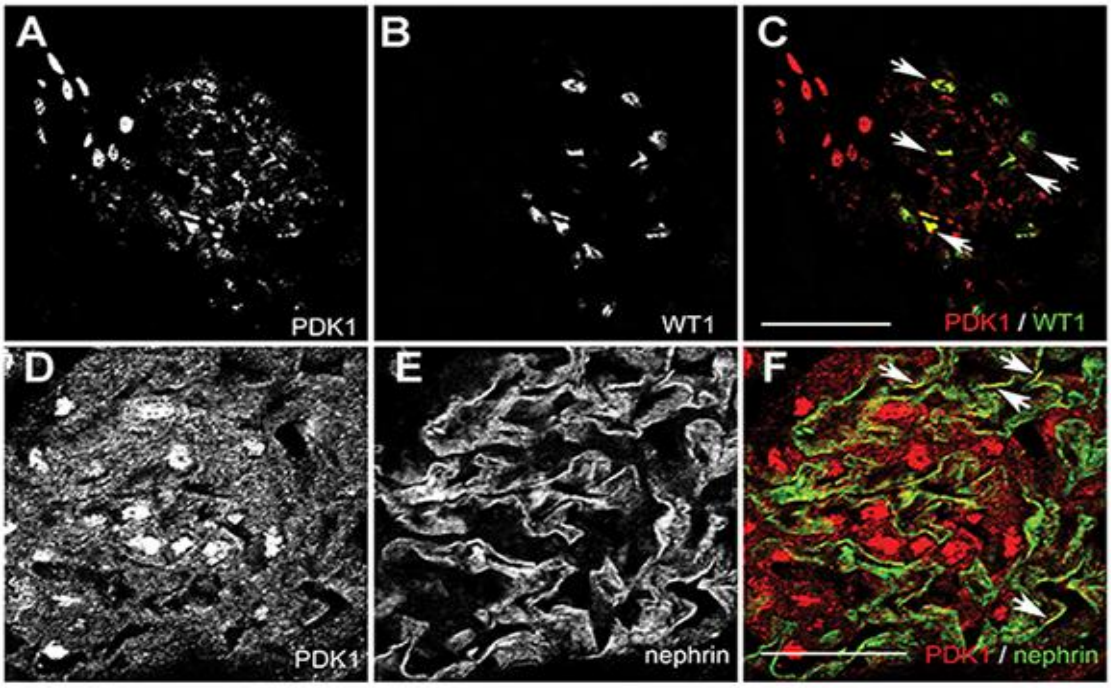

Figure 9. PDK1 is expressed in glomerular podocytes. (**A-C**) Rat kidney sections stained for PDK1 (**A**) and WT1 (**B**) show that PDK1 localizes in nuclei (arrows) in podocytes as visualized in the merged image (**C**). (**D-F**) Double labeling of PDK1 (**D**) and nephrin (**E**) indicates that PDK1 is also expressed in the podocyte cytoplasm (arrows) as visualized in the merged image (**F**). (**A-F**) Rat kidney cryosections were fixed with acetone, labeled with anti-PDK1, anti-WT1 and anti-nephrin IgGs, and examined by confocal microscopy. Scale bars: (**A-C**) 50 μm, (**D-F**) 25 μm.

We have previously shown high baseline LPS activity in sera of Finnish patients with T1D to be associated with the progression of DN (Nymark et al., 2009). To study the potential antiapoptotic role of PDK1 in LPS-induced podocyte injury, we subjected cultured podocytes to sera from normoalbuminuric T1D patients with either high (0.41 EU/ml) or low (0.20 EU/ml) LPS activity. Clinical characteristics of normoalbuminuric patients with T1D with low or high serum LPS activity are shown in Table 3. PDK1 expression was found to be lower and apoptosis induced in human podocytes exposed to sera with high LPS activity for 72 h when compared to cells treated with sera with low LPS activity (Study II, Figure 2). High glucose treatment that has previously shown to induce podocyte apoptosis (Liu et al., 2013;Susztak et al., 2006), also downregulated PDK1 expression compared to cells cultured in normal glucose or in high mannitol (osmolality control) (Study II, Supplementary Figure S1). Interestingly, blood glucose values were similar in patients with low and high serum LPS activity, indicating that high glucose did not contribute to reduced PDK1 expression or increased apoptosis.

Table 3. Patient characteristics. All patients were males. Normoalbuminuria was defined as urinary albumin excretion rate <30 mg/24 h. LPS, lipopolysaccharide; HbA1c, hemoglobin A1c; HDL, high-density lipoprotein; Duration, duration of diabetes.

LPS	Low (n=6)	High (n=6)
Age (years)	34.8 ± 3.3	33.2 ± 6.7
Duration (years)	13.8 ± 2.7	14.2 ± 3.9
LPS (EU/ml)	0.20 ± 0.01	0.41 ± 0.04*
Triglycerides (mmol/L)	1.0 ± 0.9	1.2 ± 0.6
HbA1c (%)	7.9 ± 0.8	7.7 ± 0.2
HDL-cholesterol	1.32 ± 0.12	1.34 ± 0.19
Glucose (mmol/L)	8.63 ± 2.19	5.83 ± 1.08

* $p < 0.001$, Student's t-test.

5.2.2. Knockdown of PDK1 increases apoptosis, inhibits antiapoptotic- and stimulates proapoptotic pathways in cultured human podocytes

PDK1 was knocked down in cultured podocytes in order to study the antiapoptotic role of PDK1. Two different lentiviral small hairpin RNAs (shRNAs) were used and both lowered PDK1 level compared to podocytes infected with empty vector (Study II, Figure 3A and B). Apoptosis was increased from 6% in podocytes infected with the empty vector to 22-27% in podocytes infected with PDK1 shRNAs as shown by increase in Annexin V labeled cells in flow cytometry (Figure 10 and Study II, Figure 3C).

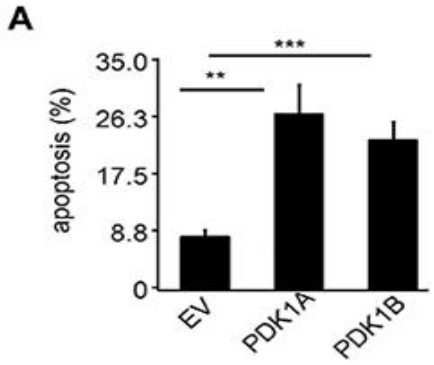

Figure 10. Knockdown of PDK1 increases apoptosis in cultured human podocytes. **A:** Flow cytometry of podocytes stained with Annexin V shows that PDK1 knockdown increases podocyte apoptosis compared to podocytes infected with empty vector (EV). The experiments were performed three times with three replicates in each experiment. The bars (**A**) show the mean expression in arbitrary units (error bars STDEV). $^{**}p < 0.01$, $^{***}p < 0.001$, Student's *t*-test.

Downregulation of PDK1 also altered the phosphorylation of apoptosis-related kinases, Akt and p38MAPK. Depletion of PDK1 reduced the phosphorylation level of Akt on S437, and increased the activation of the proapoptotic p38 MAPK pathway by inducing phosphorylation of p38 (Study II, Figure 3F,G,I and J). PDK1 knockdown also decreased the level of antiapoptotic BCL-2 and increased the level of proapoptotic BAX indicating that the intrinsic apoptotic pathway is also involved in the antiapoptotic PDK1 pathway in podocytes (Study II, Figure 3H and K).

5.2.3. PDK1 expression is downregulated in the glomeruli of diabetic Zucker rats and patients with T2D, and in podocyte injury models *in vitro*

Next, we analyzed whether PDK1 expression is reduced in the glomeruli of insulin resistant obese Zucker rats (Chua et al., 1996). PDK1 expression was found to be lower in obese Zucker rat glomeruli in both 12 and 40 weeks old rats when compared to lean controls (Study II, Figure 4A,B,D and E). There was no significant apoptosis in 12 weeks old obese rat glomeruli (Study II, Figure 4C). 40 weeks old rats showed increased glomerular apoptosis (Study II, Figure 4F). PDK1 expression was also lower in patient glomeruli with T2D compared to controls (Study II, Figure 4G,H and I). The patients with diabetes did not have clinical nephropathy, and histopathological analysis revealed no signs of DN.

We also investigated the expression of PDK1 in podocyte injury models *in vitro* using PA. PA is a toxin that induces experimental minimal change nephrosis in rats (Marshall et al., 2006). PA-

treatment decreased the expression level of PDK1 and induced apoptosis 48 hours after exposure (Figure 11).

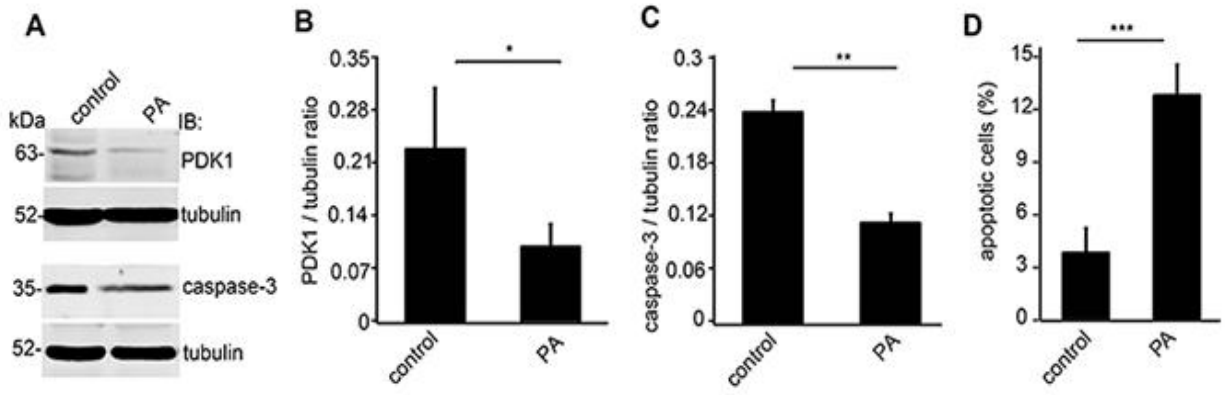

Figure 11. PA-treatment downregulates PDK1 expression and induces apoptosis in cultured human podocytes. **A:** Representative immunoblot for PDK1 and caspase-3 after PA-treatment. Tubulin is included as a loading control. **B**: Quantification of PDK1 after PA-treatment shows a decrease in PDK1 expression. **C:** Quantification of caspase-3 after PA-treatment shows a decrease in caspase-3 expression level suggesting induction in podocyte apoptosis. **D:** Flow cytometry of cultured human podocytes stained with annexin V and 7-AAD double labeling, where annexin V is used as an apoptosis and 7-AAD as a necrosis marker, confirms that PA-treatment increase podocyte apoptosis. The experiments were performed three times with three replicates in each experiment. The bars (**B**, **C** and **D**) show the mean expression in arbitrary units (error bars STDEV). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, Student's *t*-test.

5.2.4. Inhibition of the TLR pathway prevents LPS-induced downregulation of PDK1, induction of apoptosis *in vivo* and *in vitro*, and podocyte foot process widening

Toll-like receptors (TLRs), including TLR4, which acts as a receptor for LPS, are known to be expressed in podocytes (Banas et al., 2008;Poltorak et al., 1998;Srivastava et al., 2013). To study, whether inhibition of the TLR pathway could reduce LPS-induced downregulation of PDK1 and apoptosis of podocytes, cultured human podocytes were treated with GIT27 before addition of LPS (100 ng/ml) to the media. GIT27 is an active immunomodulatory agent that inhibits TLR4 and TLR2/6 signaling pathways. GIT27 and LPS co-treatment prevented LPS-induced downregulation of PDK1 and induction of podocyte apoptosis (Study II, Figure 5).

To confirm that inhibition of the TLR pathway reduces the LPS-induced apoptosis and dowregulation of PDK1 expression *in vivo*, we used LPS to induce podocyte injury and proteinuria in BALB-C mice. Mice were treated with either GIT27 or its vehicle 24 h prior to and 2 h after LPS challenge. Albuminuria, PDK1 expression and kidney morphology were analyzed 24 h after the LPS challenge.

LPS increased urinary albumin excretion, decreased glomerular expression of PDK1 and increased tubular apoptosis (Study II, Figure 6). GIT27 treatment prevented the decrease in the expression of PDK1 and the increase in apoptosis (Study II, Figure 6). GIT27 also decreased urinary albumin excretion, but this did not reach statistic significance. Electron microscopy (EM) revealed irregular widening of the podocyte foot processes in the LPS-treated mice when compared to control mice and mice treated with LPS and GIT27 (Study II, Figure 7A-E). Confirming the protective effect of GIT27, we found that the level of slit diaphragm protein podocin, essential for podocyte survival (Jefferson et al., 2008), was also reduced in LPS-treated mice (Study II, Figure 7E and F). LPS-induced decrease of podocin was prevented by GIT27 co-treatment.

5.2.5. Inhibition of the TLR pathway restores the expression of PDK1 and reduces podocyte apoptosis induced by sera from T1D patients with high LPS activity

To confirm that high serum LPS activity in T1D patients causes downregulation of PDK1, we treated cultured human podocytes with GIT27 for 2 h before exposing the cells to the media with sera from normoalbuminuric T1D patients with high LPS activity. As above, serum with high LPS activity downregulated PDK1 expression and induced podocyte apoptosis when compared to cells treated with the sera with low LPS activity. Co-treatment with GIT27 was able to prevent the downregulation of PDK1 and induction of apoptosis induced by high LPS activity (Study II, Figure 8). The data indicate that high LPS activity downregulates PDK1 expression and induces apoptosis in podocytes, and this can be prevented with GIT27 co-treatment (Study II, Figure 8).

5.3. Study III: Cyclin-dependent kinase 2 protects podocytes from apoptosis

5.3.1. CDK2 is downregulated in the glomeruli of diabetic rats, after high glucose treatment and in podocyte injury models *in vitro*

CDK2, a protein that is believed to play an essential role in cell cycle, is widely expressed in human tissues. Although Hiromura et al. (2001) showed by Western blot that CDK2 is expressed in proliferating and differentiated mouse podocytes *in vitro* (Hiromura et al., 2001), its localization and function in podocytes remains unclear. Therefore, we wanted to first confirm its expression in glomeruli by immunoblotting of isolated rat glomerular and tubular fractions and found that CDK2 is expressed in both glomeruli and tubules (Study III, Figure 1A). Localization of CDK2 in podocytes

was studied by immunofluorescence microscopy of cultured human podocytes, indicating that CDK2 is concentrated in the nuclei in podocytes (Study III, Figure 1B). We used immunofluorescense staining of perfused mouse kidney paraffin sections to confirm CDK2 expression in the nuclei of glomerular podocytes (Figure 12 and Study III, Figure 1D-I).

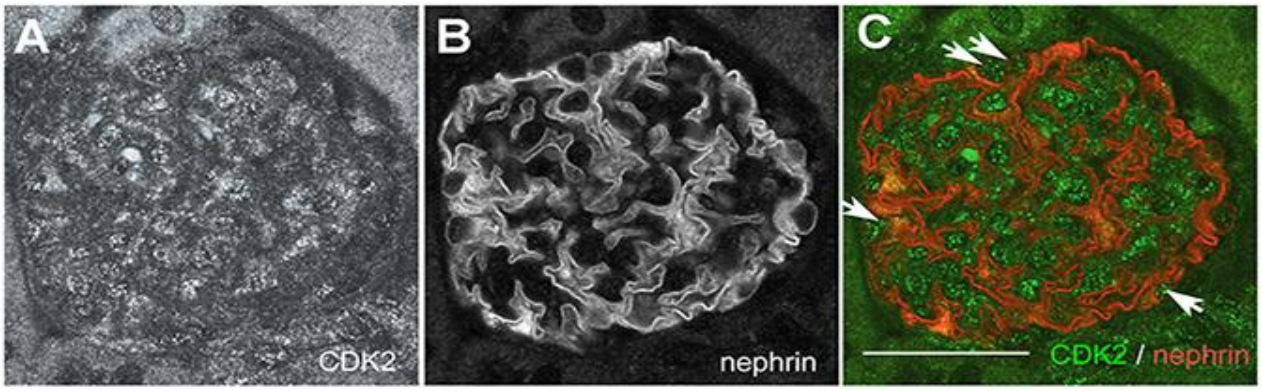

Figure 12. CDK2 is expressed in glomerular podocytes. (**A**-**C**) Double labeling of CDK2 (**A**) and nephrin (**B**) indicates that CDK2 is expressed in the nuclei (arrows) of podocyte as visualized in the merged image (**C**). (**A**-**C**) Perfused mouse kidney paraffin sections were labeled with anti-CDK2 and anti-nephrin IgGs, and examined by confocal microscopy. Scale bar: 25 μm.

To study the role of CDK2 in diabetes and DN, we studied the expression level of CDK2 in the glomeruli of insulin resistant obese Zucker rats and found that the expression level of CDK2 was lower in both 12 weeks and 40 weeks old Zucker rats when compared to lean controls (Study III, Figure 2). Blood glucose and urinary values of Zucker rats are described in Table 3.

Next, we investigated the expression of CDK2 in podocyte injury models *in vitro* using PA and LPS. LPS is known to induce septic like symptoms and proteinuria in mice and rats (Reiser et al., 2004;Remick et al., 2000). Both PA- and LPS-treatments decreased the expression level of CDK2 and induced apoptosis 48 hours after exposure (Study III, Figure 3). Treatment of human podocytes with high glucose (72 h) also downregulated CDK2 expression compared to cells cultured in normal glucose.

5.3.2. Inhibition of the TLR pathway prevents downregulation of CDK2 and induction of podocyte apoptosis induced by LPS

Next we studied whether TLR pathway inhibitor, GIT27 can also rescue LPS-induced downregulation of CDK2. LPS-treatment reduced the expression of CDK2 and induced apoptosis in cultured human

podocytes and GIT27 co-treatment was able to prevent both the downregulation of CDK2 and induction of apoptosis induced by LPS-treatment (Study III, Figure 4A-C).

To study whether T1D patient serum with high LPS activity also reduces the expression of CDK2, we treated cultured human podocytes with sera from normoalbuminuric patients with high or low LPS activity with or without GIT27 co-treatment (patient data is described in Table 4). Treatment with human sera with high LPS activity decreased the expression level of CDK2 and induced apoptosis (Study III, Figure 4D-F). Co-treatment with GIT27 prevented LPS-induced downregulation of CDK2 expression and induction of apoptosis confirming that CDK2 downregulation is mediated by endotoxins in treatment with human serum with high LPS activity (Study III, Figure 4D-F).

5.3.3. TLR Pathway blockage restores the glomerular expression of CDK2 and prevents podocyte foot process widening induced by LPS in mice *in vivo*

To confirm that LPS induced the downregulation of CDK2 and to study whether LPS-induced dowregulation of CDK2 expression can be rescued by TLR pathway inhibition *in vivo,* we used LPS with or without GIT27 co-treatment in BALB-C mice as described in Study II. LPS decreased also the expression of CDK2 in mouse kidneys, and in mouse glomeruli as visualized by immunoblotting and immunohistochemistry, respectively (Study III, Figure 5A and B). In addition, co-treatment with GIT27 prevented downregulation of CDK2 (Study III, Figure 5A and B). GIT27 treatment also prevented the increase in LPS induced apoptosis in the mouse kidneys, but this did not reach statistical significance due to due to individual variation in the treatment groups (Figure 13).

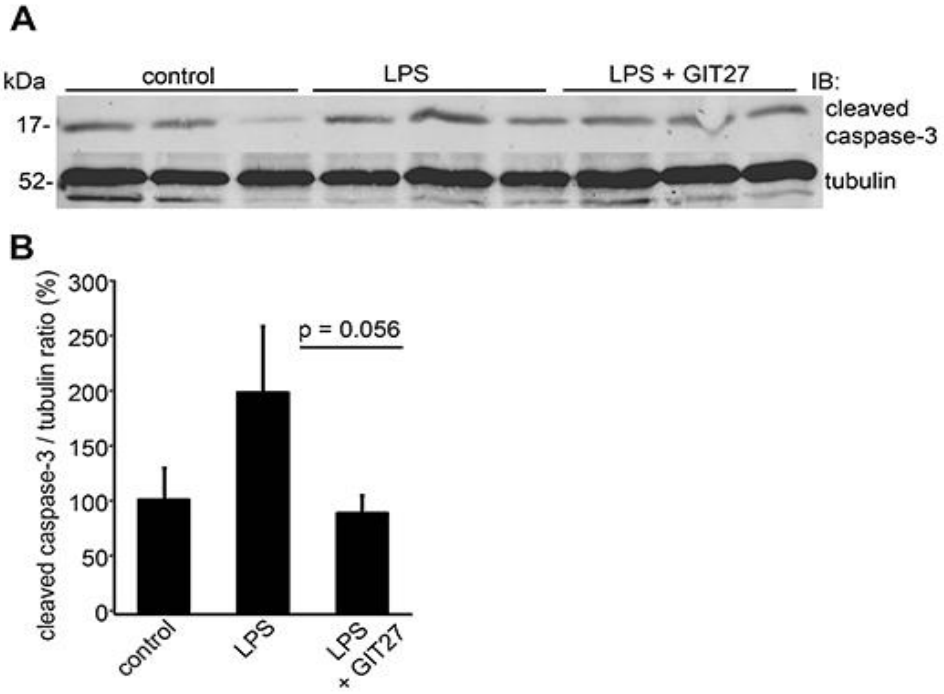

Figure 13. Inhibition of the TLR pathway prevents LPS-induced apoptosis in mouse kidneys. **A:** Representative immunoblot for cleaved caspase-3 in control, LPS-treated and LPS- and GIT27-treated mouse kidney cortical lysates. Tubulin is included as a loading control. **B:** Quantification of cleaved caspase-3 in mouse kidney cortical lysates shows that co-treatment of mice with LPS and GIT27 prevents LPS-induced apoptosis, although this did not reach statistical significance (n= 6 per treatment group).

5.3.4. Knockdown of CDK2 increases apoptosis, inhibits the antiapoptotic and stimulates the proapoptotic pathways in cultured human podocytes

CDK2 was knocked down in cultured podocytes using two different lentiviral small hairpin RNAs (shRNAs) in order to confirm the antiapoptotic role of CDK2 in podocytes. Both shRNA constructs lowered CDK2 protein level up to 85% 48 h after infection compared to podocytes infected with empty vector (Study III, Figure 6A and B). Knockdown of CDK2 induced podocyte apoptosis, as shown by flow cytometry with annexin V labeling, when compared to podocytes infected with empty vector (Figure 14 and Study III, Figure 6C).

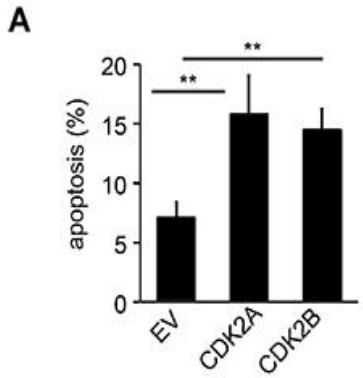

Figure 14. Knockdown of CDK2 increases apoptosis in cultured human podocytes. **A:** Flow cytometry of podocytes stained with Annexin V shows that CDK2 knockdown increases podocyte apoptosis compared to podocytes infected with empty vector (EV). The experiments were performed three times with three replicates in each experiment. The bars (**A**) show the mean expression in arbitrary units (error bars STDEV). $**p < 0.01$, Student's *t*-test.

CDK2 knockdown also had an effect on the molecular pathways involved in apoptosis, as phosphorylation of Akt was reduced and phosphorylation of p38MAPK was induced after CDK2 knockdown (Study III, Figure 7A-D). Furthermore, CDK2 knockdown also reduced the expression level of antiapoptotic BCL-2 and increased the expression level of proapoptotic BAX indicating that CDK2 influences the intrinsic apoptotic pathway in podocytes (Study III, Figure 7E and F).

5.3.5. CDK2 knockdown reduces PDK1 expression and knockdown of PDK1 reduces CDK2 expression

Since downregulation of both PDK1 and CDK2 induced podocyte apoptosis and reduced Akt phosphorylation, we next hypothesized that both of these proteins are activators of the Akt cell survival pathway and they are linked to each other. To test this hypothesis, we knocked down either CDK2 or PDK1 using lentiviral shRNAs. CDK2 knockdown reduced the expression of PDK1 suggesting that CDK2 affects the PI3K/Akt –mediated cell survival pathway by regulating PDK1 expression (Study III, Figure 8A and B). Knockdown of PDK1, in turn, reduced the expression of CDK2 suggesting a regulatory loop between CDK2 and PDK1 (Study III, Figure 7C and D).

6. DISCUSSION

6.1. Function of SHIP2 in Akt signaling pathway and apoptosis in podocytes

We identified SHIP2 as a new interaction partner of CD2AP, an adaptor protein critical for podocyte function in maintaining the GFB (Shih et al., 1999). CD2AP is essential for podocyte function, since damage to CD2AP not only affects the function of SDs, but also directly damages the podocyte cytoskeleton leading to pathological changes such as podocyte loss (Schwarz et al., 2001). In addition, CD2AP expression was decreased in albumin-induced podocyte apoptosis (He et al., 2011). CD2AP also stimulates PI3K-dependent Akt signaling pathway (Huber et al., 2003), and lack of CD2AP enhances the activation of the proapoptotic p38 MAPK pathway by TGF-ß1 (Schiffer et al., 2004). Since SHIP2 is a negative regulator of insulin signaling in adipocytes (Dyson et al., 2005), our finding showing that SHIP2 interacted with CD2AP, suggest that SHIP2 might be involved in Akt dependent insulin signaling also in podocytes. Although SHIP2 has previously shown to be involved in the regulation of food intake and expressed in elevated levels in adipose tissue, skeletal muscle and in the hypothalamus of db/db mice (Ichihara et al., 2013), its expression and function in the kidney has not been studied previously.

SHIP2 is a lipid phosphatase belonging to the inositol 5-phosphatase family acting on phosphoinositol(3,4,5)trisphosphate (PI(3,4,5,)P_3) (Dyson et al., 2005). SHIP2 hydrolyses the 5-position phosphate from PI(3,4,5,)P_3 to form phosphoinositol(3,4)biphosphate (PI(3,4,)P_2) and therefore negatively regulates the PI3K pathway (Blero et al., 2001;Pesesse et al., 1997;Taylor et al., 2000;Wisniewski et al., 1999). In addition, SHIP2 has been reported to be phosphorylated in response to numerous growth factors including insulin (Habib et al., 1998;Ishihara et al., 1999;Muraille et al., 1999;Wisniewski et al., 1999). Since podocytes have shown to be insulin responsive cells (Coward et al., 2005) and SHIP2 was reported to negatively regulate PI3K pathway, the major cell survival pathway in podocytes (Huber et al., 2003), we hypothesized that SHIP2 would be involved in Akt signaling pathway and apoptosis also in podocytes.

First, SHIP2 was found to be expressed in glomerular podocytes. In addition, the intracellular interaction of CD2AP and SHIP2 was confirmed. The catalytic activity of SHIP2 has been suggested to be mediated by enhanced tyrosine phosphorylation (Batty et al., 2007;Pesesse et al., 2001), however, Edimo et al. (2012) suggested, based on other publications, that several key properties of

SHIP2 are regulated by serine/threonine phosphorylation (Edimo et al., 2012). In addition to the catalytic activation of SHIP2, it has been shown to be relocated to the plasma membrane upon growth factor stimulation (Pesesse et al., 2001). Our data demonstrate that SHIP2 is translocated to the plasma membrane in response to insulin in podocytes. However, the interaction of CD2AP and SHIP2 was not dependent on insulin stimulus. CD2AP bound only to the non-tyrosine-phosphorylated form of SHIP2 independently of insulin stimulation suggesting that the interaction of CD2AP and SHIP2 is not predominantly due to regulation of insulin signaling.

In cultured human podocytes, overexpression of SHIP2 led to decreased Akt phosphorylation. Supporting our finding, SHIP2 has been shown to downregulate insulin-induced Akt activation in adipocytes (Wada et al., 2001). Previous finding, showing that overexpression of SHIP2 in Chinese hamster ovary cells overexpressing the insulin receptor led to a decrease of the insulin-dependent PI(3,4,5)P3 production as well as decrease in Akt and MAPK activation (Blero et al., 2001) together with our results indicating, that SHIP2 overexpression downregulates the PI3K pathway in podocytes, suggests that SHIP2 may be involved in the development of impaired insulin signaling in podocytes.

Impaired insulin response might be involved in podocyte loss in DN, since Akt phosphorylation has previously been shown to act as a survival signal in podocytes and to protect them from apoptosis (Huber et al., 2003). Given the importance of Akt signaling pathway in cell survival, and since SHIP2 reduced Akt signaling in podocytes, we studied the role of SHIP2 in podocyte apoptosis. Indeed, in addition to decreased Akt activation, overexpression of SHIP2 also increased podocyte apoptosis, suggesting that SHIP2 could contribute to development of the glomerular injury by downregulating the Akt survival pathway.

SHIP2 expression in podocyte injury model *in vitro* was studied to confirm the involvement of SHIP2 in podocyte apoptosis. Recently it was shown that overexpression of angiopoietin-like3 (Angptl3), a protein that induces F-actin arrangement in podocytes (Lin et al., 2013), accelerated the PA-induced podocyte loss *in vitro* (Dai et al., 2015). Since SHIP2 overexpression also induced podocyte apoptosis and SHIP2 has previously shown to regulate the maintenance and remodeling of actin structures in HeLa cells (Prasad and Decker, 2005), we studied whether SHIP2 expression increases in PA-induced podocyte injury model. PA is a podocyte toxin used in rats to induce experimental minimal change disease progressing to FSGS (Marshall et al., 2006). We observed that SHIP2 expression and podocyte apoptosis were upregulated in PA-induced podocyte injury *in vitro*. Wang et al. (2015) demonstrated recently that VEGF promotes podocyte survival against PA-induced apoptosis by

enhancing PI3K/Akt pathway (Wang et al., 2015). The increased expression of SHIP2 in PA-induced podocyte injury together with data showing that the SHIP2 overexpression downregulates the PI3K/Akt pathway in podocytes, implicates that upregulation of SHIP2 could be one possible mechanism inducing podocyte apoptosis in podocyte injury.

In addition to cell survival, Akt signaling is involved in glucose uptake, where PI3K increases intracellular PI(3,4,5,)P_3 at the plasma membrane leading to the activation of Akt and PDK1, which is required for insulin-induced action such as translocation of GLUT4 to the plasma membrane (Dugani and Klip, 2005;Farese et al., 2005). Activation of PI3K/Akt signaling pathway is involved in the regulation of insulin signaling as Akt2 knock-out mice are insulin resistant and diabetic (Cho et al., 2001;Garofalo et al., 2003;Canaud et al., 2013). Previous discoveries showing that SHIP2 is expressed in elevated levels in skeletal muscle and adipose tissue of db/db mice (Hori et al., 2002), combined with our results showing decreased Akt signaling after SHIP2 overexpression, suggests that SHIP2 might be involved also in glucose uptake in podocytes

6.2. PDK1 and CDK2 regulate PI3K/Akt cell survival pathway and apoptosis in podocytes

Given the pivotal role of the PI3K-dependent Akt signaling pathway in the regulation of cell survival, we next studied molecules known to be involved in the modulation of its activation, both upstream and downstream of Akt. We concentrated on PDK1, a serine/threonine kinase that functions downstream of PI3K and upstream of Akt and is known to act as a major regulatory point in Akt signaling (Keledjian et al., 2012) and CDK2, a S-phase cyclin-dependent kinase, that has been shown to be phosphorylated by Akt *in vitro* in 293T cells (Maddika et al., 2008). In addition, in HeLa cells, CDK2 was shown to promote the activation of Akt by phosphorylating residues S477 and T479 under cell cycle progression (Liu et al., 2014).

In podocytes, the expression and function of PDK1 had not been studied before. First, PDK1 was confirmed to be expressed in glomerular podocytes. Since PDK1 was shown to be involved in regulation of Akt signaling (Keledjian et al., 2012), we hypothesized that it may be involved in the regulation of apoptosis in podocytes. Previously, it has been shown that PDK1 knockout mice die *in utero*, but mice that lack PDK1 specifically in the pancreatic β cells develop progressive hyperglycemia as a result of the loss of islet mass (Hashimoto et al., 2006;Lawlor et al., 2002).

Even though Hiromura et al. (2001) showed that CDK2 is expressed in both proliferating and differentiated mouse podocytes *in vitro*, its localization and function in podocytes remains unclear. We found that CDK2 is expressed in glomerular podocytes, although previous studies have mainly shown negligible expression of CDK2 in healthy glomerulus (Shankland et al., 1997;Wang et al., 2004). The difference in CDK2 expression in previous studies and in our study may be due to different experimental conditions, or antibodies used. In addition, previous studies addressing the role of CDK2 in the kidney have concentrated on proliferative podocyte injury models. In experimental glomerulonephritis, where podocytes re-enter the cell cycle and start to proliferate, inhibition of CDK2 has been shown to improve renal function by inhibiting proliferation of podocytes (Griffin et al., 2005). Inhibition of CDK2 by roscovitine has also been shown to decrease mesangial and glomerular endothelial cell proliferation in passive Heymann nephritis, a model of membranous nephropathy, without aggravating podocyte damage (Milovanceva-Popovska et al., 2005). Since mature podocytes are terminally differentiated and therefore have a limited capacity to proliferate, the role of CDK2 in healthy, nonproliferating podocytes and nonproliferative glomerular diseases, such as DN, remains unclear. Furthermore, CDK2 activity is regulated by several factors, as CDK2 is controlled by cyclin-dependent kinase inhibitors (Hiromura et al., 2001;Shankland et al., 1997) and in cell cycle, CDK2 is activated by complex formation with cyclin E, or A (Bartova et al., 2004).

To study whether PDK1, or CDK2 are involved in the regulation of PI3K-dependent Akt signaling pathway and apoptosis in podocytes, we knocked down either PDK1 or CDK2 in cultured human podocytes. Previous studies have shown that in addition to Akt (Bridgewater et al., 2005;Chen et al., 2009) and 38p MAPK (Chen et al., 2009) pathways, BCL-2 and BAX are involved in podocyte apoptosis (Lee et al., 2009;Sun et al., 2009a). Indeed, knockdown of either PDK1, or CDK2 in cultured human podocytes decreased the activation of antiapoptotic Akt and the expression of BCL-2, and in addition, stimulated the proapoptotic p38 MAPK activation and increased the expression of BAX. Since knockdown of either PDK1 or CDK2 led also to an increase in apoptosis, we hypothesized that both PDK1 and CDK2 protect podocytes from apoptosis by stimulating the PI3K-dependent Akt signaling pathway.

Supporting our data for the role of PDK1 as an antiapoptotic protein, inhibition of the Akt pathway with a specific PDK1 inhibitor has previously been shown to induce apoptosis of human mast cells (Sawamukai et al., 2007). In addition, induction of PDK1 activity has been shown to decrease apoptosis by activating Akt in intestinal epithelial cells (Keledjian et al., 2012). Supporting our finding for CDK2 as protein protecting podocytes from apoptosis, inhibition of CDK2 in HeLa cells

has been shown to decrease Akt phosphorylation (Liu et al., 2014). In line with this, CDK2 associated with cyclin A2 and Akt phosphorylation was inhibited in olfactory bulbs of brain-specific cyclin A2 knockout mice leading to increased activation of caspase-3 and cellular apoptosis (Liu et al., 2014).

To further confirm the role of PDK1 and CDK2 in podocyte apoptosis, we used PA-induced *in vitro* podocyte injury model. PA was previously shown to increase BAX and decrease BCL-2 expression in cultured mouse podocytes (Remick et al., 2000). We observed that treatment of podocytes with the toxin PA induced podocyte apoptosis and downregulated both PDK1 and CDK2, suggesting that both PDK1 and CDK2 have a role in PA-induced podocyte apoptosis.

Since PA is a toxin used only in experimental conditions to induce experimental minimal change disease or FSGS (Marshall et al., 2006), we wanted to study the expression of PDK1 and CDK2 also in conditions closer to diabetic diseases *in vivo*. Since increased podocyte apoptosis has previously been shown *in vitro* after high glucose-treatment (Susztak et al., 2006), and also *in vivo* in both Akita mice with T1D and in db/db mice with T2D (Susztak et al., 2006), we used high glucose to study whether PDK1 and CDK2 are involved in podocyte injury induced by high glucose. In cultured podocytes, high glucose treatment decreased both PDK1 and CDK2 expression (Studies I and II). Wang et al. (2014) showed recently, that high glucose decreased BCL-2 and increased BAX protein level and induced podocyte apoptosis, and knockdown of Akt accelerated these effects (Wang et al., 2014). In the same study, high glucose induced podocyte apoptosis was prevented by PI3K/Akt pathway activation (Wang et al., 2014). In addition, Lee et al. (2009) demonstrated that BAX and active caspase-3 expressions were upregulated in diabetic rat glomeruli and in cultured mouse podocytes treated with high glucose (Lee et al., 2009). Our findings together with these recent discoveries suggest that PDK1 and CDK2 may mediate glucose-induced podocyte apoptosis through PI3K/Akt pathway.

In addition to PA- and high glucose treatment, LPS-treatment has shown to be involved in the development of albuminuria and podocyte apoptosis. In fact, LPS-treatment of mice was shown to lead to general inflammation and diabetes and induction of albuminuria (Cani et al., 2007;Cunningham et al., 2000;Reiser et al., 2004).We treated cultured podocytes with human sera with high LPS, since it has been shown that high serum LPS activity in normoalbuminuric patients with T1D predicts the progression of DN (Nymark et al., 2009). Treatment of podocytes with sera with high LPS activity from patients with T1D downregulated both PDK1 and CDK2, and induced podocyte apoptosis indicating that these proteins may have a role in maintaining normal podocyte

function, since incidence of bacterial infections in Finnish patients with T1D has been shown to correlate with the severity of DN (Simonsen et al., 2015). However, there might be some other factors in human sera in addition to LPS that could contribute to downregulation of PDK1 and CDK2. Nevertheless, high glucose did not contribute to increased apoptosis or decreased PDK1, or CDK2 expression in treatment with human sera with high LPS, since blood glucose values were similar in patients with low and high serum LPS. In addition to human sera with high LPS activity, treatment with exogenously added LPS decreased CDK2 and PDK1 expressions and induced podocyte apoptosis. In line with this, administration of LPS to mice *in vivo* also reduced the levels of CDK2 and PDK1 in glomeruli and induced apoptosis in the kidneys. LPS-treatment of mice induced podocyte foot process widening and albuminuria.

To find possible treatment options for LPS-induced podocyte injury, we blocked the TLR4 pathway since LPS binds to TLR4 also in podocytes (Banas et al., 2008;Reiser et al., 2004;Srivastava et al., 2013). Mice that lack TLR4 (Hoshino et al., 1999), or mice in which TLR4 is mutated (Poltorak et al., 1998;Qureshi et al., 1999), are low responders to LPS. We used immunomodulatory agent GIT27 to inhibit the TLR4 pathway. GIT27 treatment prevented the LPS-induced and high serum LPS activity induced downregulations of CDK2 and PDK1 both, *in vitro* and *in vivo*. GIT27 treatment also prevented LPS-induced apoptosis *in vitro* and *in vivo* and podocyte foot process effacement in LPS-treated mice. Due to the non-nephrotoxic feature of GIT27 (Cha et al., 2013;Min et al., 2014), it may be clinically potential in the treatment of DN. Since GIT27 is known to inhibit also TLR2 (Stojanovic et al., 2007) that is expressed in podocytes (Srivastava et al., 2013) and linked with diabetes (Dasu et al., 2008;Dasu and Jialal, 2011;Devaraj et al., 2008), GIT27 might protect podocytes also by inhibition of the TLR2 pathway in addition to the TLR4 pathway.

6.3. SHIP2, PDK1 and CDK2 in T2D *in vivo*

Since SHIP2, PDK1 and CDK2 were found to be involved in the regulation of Akt cell survival pathway and podocyte apoptosis *in vitro*, we further studied these proteins in animal models and human patients with T2D. We found SHIP2 expression to be slightly increased in glomeruli of db/db mice, a known model of insulin resistance characterized by hyperphagia and obesity (Chua et al., 1996). Insulin resistance has been previously shown in podocyte cell line derived from db/db mice compared to podocyte line from control mice (Tejada et al., 2008), and SHIP2 has been linked to insulin resistance and diabetes in SHIP2 deficient mice (Clement et al., 2001). Since SHIP2 was upregulated in glomeruli of db/db mice and Suztak et al. (2006) showed increase in podocyte

apoptosis in db/db mice before increase in urinary albumin excretion (Susztak et al., 2006), SHIP2 may be one of the factors inducing the development of insulin resistance and injury of podocytes in these mice.

We further observed that SHIP2 expression was increased and PDK1 and CDK2 decreased in glomeruli of insulin resistant obese Zucker rats already at 12 weeks of age compared to lean controls. Interestingly, although it has been previously reported that Zucker rats do not develop proteinuria or glomerular apoptosis at 12 weeks of age (Cani et al., 2007;Coimbra et al., 2000;Dasu et al., 2008;Dasu and Jialal, 2011), we saw trend in apoptosis already in 12 week old Zucker rats. Since these rats had not yet developed proteinuria at 12 weeks of age, and podocytes we saw trend in apoptosis already in 12 weeks old Zucker rats, although this did not reach statistical significance are terminally differentiated cells, even a small increase in apoptosis could contribute to glomerular injury. Therefore SHIP2, PDK1 and CDK2 might be associated with the development of podocyte apoptosis already at early stage. Further supporting our findings, glomerular apoptosis was shown in the kidneys of patients with DN, already in early nephropathy (Verzola et al., 2007), and PDK1 was downregulated in the glomeruli of patients with T2D who had not yet developed clinical nephropathy.

More data is needed to conclude how Akt signaling pathway is regulated in podocytes, however, our data demonstrate that SHIP2 downregulates PI3K-dependent Akt signaling pathway, PDK1, and also CDK2 activates Akt. Furthermore, knockdown of PDK1 reduced CDK2 expression, and knockdown of CDK2 reduced PDK1 expression suggesting a regulatory loop between these two proteins. Given the central role of the PI3K-dependent Akt signaling pathway in regulating cell survival, we concluded that SHIP2, PDK1 and CDK2 regulate apoptosis in podocytes by modulating the activity of the PI3K-dependent Akt signaling pathway (Figure 15).

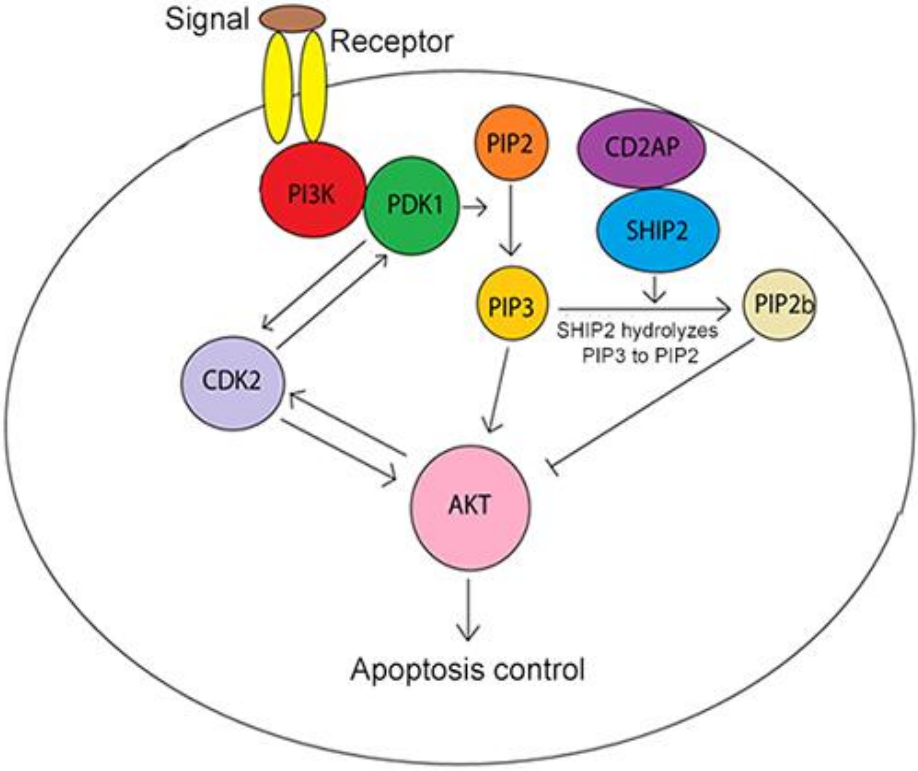

Figure 15. PI3K-dependent Akt signaling in podocyte apoptosis. PI3K: phosphoinositide 3-kinase; PDK1: 3-phosphoinositide dependent kinase 1; CDK2: cyclin-dependent kinase 2; CD2AP: CD2-associated protein; SHIP2:SH2-domain-containing inositol polyphosphate 5-phosphate 2; PIP2: phosphoinositol(4,5)biphosphate; PIP3: phosphoinositol(3,4,5)trisphosphate; PIP2b: phosphoinositol(3,4)biphosphate.

7. SUMMARY AND FUTURE PERSPECTIVES

The studies in this thesis provide new discoveries in regulation of podocyte apoptosis in the development of DN, concentrating on three molecules expressed in podocytes, SHIP2, PDK1 and CDK2. Biochemical methods were used for characterization of protein-protein interactions. In addition, cell culture experiments and diabetic and proteinuric animal models were used to study the expression and function of SHIP2, PDK1 and CDK2 in podocyte apoptosis.

Study I demonstrated that SHIP2 is expressed in glomerular podocytes and that SHIP2 is an interaction partner of CD2AP. The interaction of CD2AP and SHIP2 was not predominantly due to regulation of insulin signaling, since CD2AP bound only to the non-tyrosine-phosphorylated form of SHIP2 independently of insulin stimulation. Overexpression of SHIP2 in cultured human podocytes downregulated insulin signaling by reducing Akt phosphorylation and induced apoptosis. The expression of SHIP2 was found to be upregulated in the glomeruli of diabetic Zucker rats prior to the development of albuminuria suggesting a role for SHIP2 in the development of podocyte injury. In addition, PA-treatment of podocytes upregulated SHIP2 expression and induced apoptosis. In conclusion, SHIP2 promotes impaired insulin signaling and podocyte apoptosis by downregulating the Akt signaling pathway. However, it is still unknown whether LPS-treatment upregulates SHIP2, since LPS induces podocyte apoptosis. In addition, further studies are needed to define whether downregulation of SHIP2 using shRNA lentiviral infections, or inhibition of its activity using specific SHIP2 inhibitor either in podocytes *in vitro*, or diabetic animal models *in vivo* can protect podocytes from developing insulin resistance and apoptosis. Therefore, in the future, inhibition of SHIP2 could produce significant benefits in treatment of diabetes and its kidney complications.

Studies II and III showed that glomerular podocytes express PDK1 and CDK2. In addition, both PDK1 and CDK2 were downregulated in diabetic rats before the onset of proteinuria, suggesting that PDK1 and CDK2 are associated with podocyte injury and development of DN. Supporting these results, PDK1 was also downregulated in the glomeruli of patients with T2D prior to the onset of proteinuria. Further supporting our data, treatment of podocytes with high glucose suppressed PDK1 and CDK2 expression. In addition, treatment of human podocytes with sera from normoalbuminuric T1D patients with high LPS activity reduced both PDK1 and CDK2 expression and induced apoptosis. In line with this, LPS-treatment *in vitro* and *in vivo* downregulated both PDK1 and CDK2, and induced apoptosis, which was prevented by inhibition of the TLR signaling pathway using

GIT27. Knockdown of either PDK1 or CDK2 in cultured podocytes increased apoptosis, and downregulated Akt phosphorylation, BCL-2 protein and upregulated the proapoptotic p38 MAPK pathway and BAX expression confirming their role in podocyte apoptosis. In addition, knockdown of PDK1 downregulated CDK2 and CDK2 knockdown downregulated PDK1 expression suggesting a regulatory loop between these proteins. The results of these studies suggest that PDK1 and CDK2 protect podocytes from apoptosis by activating the Akt survival pathway, and that LPS-induced podocyte injury can be prevented by blocking the TLR pathway and by preventing downregulation of PDK1 and CDK2. Still, more investigations are required to define the specific role of PDK1 and CDK2 in regulating podocyte apoptosis. For instance, would co-treatment with high glucose and LPS enhance the downregulation of PDK1 and CDK2 and would knockdown of PDK1 or CDK2 in diabetic animal models *in vivo* increase the susceptibility to glomerular diseases. In addition, it is unknown whether overexpression of PDK1 or CDK2 can prevent high glucose, PA-, or LPS-induced podocyte apoptosis, and whether podocyte-specific overexpression of PDK1 or CDK2 in diabetic animal models can prevent the progression of the disease. In the future, factors that upregulate PDK1 and/or CDK2 in podocytes could be a promising treatment strategy for diabetic kidney disease.

Since the LPS-treatment used in the studies in this thesis was a short-term and high concentration treatment, that causes sepsis and albuminuria, it would be interesting to study the expression of PDK1 and CDK2 in mice with a long-term and low concentration LPS-treatment, where they develop diabetes in addition to albuminuria. Also, whether long-term GIT27 administration prevents LPS-induced diabetes and possible downregulation of PDK1 and CDK2 in these mice. In addition, since PDK1 and CDK2 where downregulated by high glucose treatment and were involved in the regulation of Akt signaling pathway, that also is involved in the regulation of insulin signaling, future investigations could study the role of PDK1 and CDK2 in insulin signaling and insulin resistance in podocytes.

Taken together, pathogenesis of DN is multifactorial and several genetic and environmental components might contribute to its development and progression. However, the precise mechanisms remain unknown. It is known that apoptosis is involved in the development of DN, but the mechanisms leading to diabetes-induced podocyte cell death are not fully understood. In this thesis work we identified SHIP2, PDK1 and CDK2 as novel regulators of podocyte apoptosis, since they were associated with several features of DN: insulin resistance, podocyte apoptosis and T2D. In addition, several factors contributed to the changes in SHIP2, PDK1 and CDK2 expression, including PA, LPS and high glucose. Given the central role of the PI3K-dependent Akt signaling pathway in

regulating cell survival, we concluded that SHIP2, PDK1 and CDK2 regulate apoptosis in podocytes by modulating the activity of the PI3K-dependent Akt signaling pathway. Therefore differences in the expression of these proteins specifically in podocytes separately, or in cooperation might lead to, or prevent the development of podocyte apoptosis and therefore diabetic kidney complications. However, many other factors may be involved in the regulation on apoptosis and Akt signaling pathway in podocytes and thereby additional studies are needed to test this hypothesis.

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